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The human infectious reservoir of falciparum malaria

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Thesis submitted in accordance with the requirements for the
degree of Doctor of Philosophy, University of London

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I, Bronner Pamplona Augusto Gonçalves, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Malaria control strategies are often targeted at individuals who suffer most morbidity, not at those driving transmission. This is in part due to our limited understanding, described in Chapter 1, of the human infectious reservoir of falciparum malaria – i.e. individuals responsible for human-to-mosquito transmission of *Plasmodium falciparum* parasites in endemic areas. This work, whose objectives are listed in Chapter 2, assessed the prevalence of infectiousness in naturally exposed human populations during dry and wet seasons. Exposure to *Anopheles* mosquitoes, a measure of transmission opportunities, was also quantified; and to determine the value of infectiousness-reducing interventions, the use of primaquine to block transmission from infectious individuals was investigated.

Experimental infections of mosquitoes were performed to determine malaria infectivity of randomly selected individuals in two villages in Burkina Faso. Molecular assays were used to quantify parasite, including gametocyte, densities. Less than 10% of the population was infectious to mosquitoes. These results are presented in a manuscript that included data from other study sites (Chapter 3).

To assess exposure to malaria vectors, bloodfed mosquitoes were collected indoors in one of the study villages in Burkina Faso. A multiplex PCR assay targeting nine human microsatellites and a gender-specific marker was used to identify the human sources of mosquito blood meals. Although there was substantial variation in the number of mosquito bites each individual received (Chapter 4), on average adults received more mosquito bites than children. This suggests that, despite their lower infectiousness, adults are major contributors to malaria transmission in endemic areas.

An efficacy trial of single low dose primaquine was performed in Burkina Faso and pre- and post-treatment infectiousness were quantified by mosquito feeding experiments to assess primaquine's infectiousness-reducing activity (Chapter 5). Individuals receiving primaquine cleared gametocytes faster than individuals who received artemether-lumefantrine alone. Feeding assays, however, suggest that artemether-lumefantrine blocks most parasite transmission after treatment administration.

In Chapter 6, these findings, and how they can inform future control strategies, are discussed.

Acknowledgements

The populations of the villages of Laye and Balonghin in Burkina Faso

My supervisor, Chris Drakeley, and co-supervisor, Teun Bousema, for giving me the opportunity to work on several unique projects and for their mentorship

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Statement of Contributions and Additional Publications

The work described here was developed at the Centre National de Recherche et de Formation sur le Paludisme (CNRFP) in Burkina Faso, at the Radboud University Medical Center (RUMC) in the Netherlands, and at the London School of Hygiene & Tropical Medicine (LSHTM) in the UK by numerous scientists, technicians, clinicians, nurses. Below is an incomplete account of my role and the roles of these different collaborators in the various studies – for the sake of brevity, despite their utmost importance, the names of those individuals who spent several months in the villages collecting and processing samples are not listed here.

Chris Drakeley (CD; my supervisor) and Teun Bousema (TB; my co-supervisor) conceived the studies presented here as part of a research programme funded by the Bill & Melinda Gates Foundation.

Chapter 1

The first chapter describes in detail the transmission of *Plasmodium falciparum* parasites. Although most of this chapter constitutes original work, the sections on xenodiagnostic surveys and human exposure to mosquitoes are based on a review led by Will Stone (RUMC) and to which I contributed.

Stone W, Gonçalves BP, Bousema T, Drakeley C. Assessing the infectious reservoir of falciparum malaria: past and future. Trends in Parasitology 2015

Chapter 2

This short chapter summarises the objectives of the different research projects presented in this thesis.

Chapter 3

In chapter 3, data from two infectivity surveys undertaken in Burkina Faso are presented. These data were used in a manuscript that also includes results of similar surveys in Kenya. These cross-sectional studies were conceived by CD and TB; I (with CD and TB) contributed to the development of the study protocols used in Burkina Faso. I also prepared and supervised field activities with Alfred Tiono (AT). Samples

from these studies were analysed by Kjerstin Lanke (KL), who is based at the RUMC. In addition to infectivity surveys, the manuscript presented in this chapter also includes data from mosquito collection studies performed in Balonghin (Burkina Faso) and Mbita (Kenya). These entomological studies were conceived by CD and TB; CD, TB, Moussa Guelbéogo and I developed the study protocol; I was also involved in field activities. A molecular assay was used to identify mosquito blood meal sources; this assay was performed at the LSHTM, most often by Lynn Grignard and less often by me. Capillary electrophoresis was performed at the MRC Clinical Sciences Centre (London). I was not involved in field activities in Kenya. I wrote the first version of the manuscript with CD, TB, and Melissa Kapulu, who is based at the Kenya Medical Research Institute (Kilifi, Kenya). I also performed the statistical analysis and generated the graphs included in this chapter.

Chapter 4

Chapter 4 uses data from the mosquito collection studies performed in Burkina Faso. Molecular assays that determine mosquito species and detect malaria infection in mosquitoes' head-thoraces were performed at the CNRFP by the entomology team. An early draft of the manuscript that was submitted for publication in October 2017, after this thesis was first submitted, is presented in this chapter; I wrote the first draft and performed the analyses included in this version of the manuscript. A revised version of the manuscript, with modifications suggested by reviewers, as well as our answers to reviewers' comments are included in the *Appendix*. In this revised version, Laith Yakob (LSHTM) performed the analysis of malaria R_0 amplification due to mosquito biting variation; and John Bradley (LSHTM) contributed to the analysis of household-level clustering of infected mosquitoes.

Chapter 5

In chapter 5, the results of a primaquine efficacy trial performed in Burkina Faso are presented. This study was conceived by CD and TB, who also wrote the original protocol. I supervised field activities, including mosquito membrane feeding experiments and sample collection and processing, with the local principal investigator (AT). Samples from this study were analysed by KL. I performed the statistical analysis, generated the graphs, and wrote, with CD, TB and AT, the first draft of the manuscript. John Bradley (LSHTM) helped with estimations of

gametocyte clearance rates and gave valuable advices on some of the comparisons performed.

Gonçalves BP, Tiono AB*, Ouédraogo A, Guelbéogo WM, Bradley J, Nebie I, Siaka D, Lanke K, Eziefula AC, Diarra A, Pett H, Bougouma EC, Sirima SB, Drakeley C, Bousema T. Single Low Dose Primaquine to Reduce Gametocyte Carriage and Plasmodium falciparum Transmission after Artemether-Lumefantrine: A Randomised, Double-Blind, Placebo-Controlled Trial. BMC Medicine 2016*

Chapter 6

While some sections of this chapter were based on published manuscripts (listed below and included in the *Appendix*), most of chapter 6 consists of original discussion on the implications of the findings presented in this document.

Gonçalves BP, Drakeley C, Bousema T. Infectivity of Microscopic and Submicroscopic Malaria Parasite Infections in Areas of Low Malaria Endemicity. Journal of Infectious Diseases 2016.

Gonçalves BP, Walker PG, Cairns M, Tiono AB, Bousema T, Drakeley C. Pregnant Women: An Overlooked Asset to Plasmodium falciparum Malaria Elimination Campaigns? Trends in Parasitology 2017

Additional publications

I also contributed to other projects that were not part of my PhD work:

Gonçalves BP, Gupta S, Penman BS. Sickle haemoglobin, haemoglobin C and malaria mortality feedbacks. Malaria Journal 2016

Pett H, Gonçalves BP, Dicko A, Nébié I, Tiono AB, Lanke K, Bradley J, Chen I, Diawara H, Mahamar A, Soumare HM, Traore SF, Baber I, Sirima SB, Sauerwein R, Brown J, Gosling R, Felger I, Drakeley C, Bousema T. Comparison of molecular quantification of Plasmodium falciparum gametocytes by Pfs25 qRT-PCR and QT-NASBA in relation to mosquito infectivity. Malaria Journal 2016

Gonçalves BP, Pett H, Tiono AB, Murry D, Sirima S, Niemi M, Bousema T, Drakeley C, Ter Heine R. Age, weight, and CYP2D6 genotype are major determinants of primaquine pharmacokinetics in African children. Antimicrob Agents Chemother. 2017

Two manuscripts in this list were included in the *Appendix* as they are relevant to the interpretation of gametocyte quantification assays, used in the studies presented in chapters 3, 4 and 5, and primaquine efficacy (Chapter 5).

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Abbreviations

ACT	Artemisinin-based combination therapy
AL	Artemether-lumefantrine
AUC	Area under the curve
ITN	Insecticide-treated bed net
MDA	Mass drug administration
NASBA	Nucleic acid sequence based amplification
PQ	Primaquine
qPCR	Quantitative polymerase chain reaction
qRT-PCR	Quantitative reverse transcription-polymerase chain reaction
RBC	Red blood cell
RDT	Rapid diagnostic test
TBI	Transmission blocking immunity
WHO	World Health Organization

Chapter 1, Malaria Transmission

Few human infections have their name derived from their transmission mechanism – *mala aria*, ‘bad air’, is one of them. Of the five malaria parasite species infecting humans, two, *Plasmodium vivax* and *Plasmodium falciparum*, are arguably major public health problems [1, 2], causing hundreds of millions of clinical cases each year [3]. This work focuses on the more virulent species, *P. falciparum*, and its transmission in endemic settings.

The complexity of malaria transmission

A new era of malaria control, focused in reducing contact between humans and malaria vectors, started when the key events of transmission were elucidated [4]. Indeed, shortly after Alphonse Laveran’s observation of what we now know as exflagellation [5], the hypothesis that mosquitoes were the source of parasites causing human malaria infection was proposed by Laveran himself and Sir Patrick Manson. By the end of the nineteenth century, Sir Ronald Ross, working in India, had shown that mosquitoes were responsible for transmitting avian malaria [6], and the Italian zoologist Battista Grassi had demonstrated that humans acquire their infections from mosquitoes: a healthy individual living in a non-endemic region became infected with malaria parasites after being exposed to experimentally-infected mosquitoes [7, 8].

Today, more than a century later, our knowledge of these transmission events is much more detailed, although far from complete. We now know that there is a non-replicating transmission blood stage, gametocyte, that develops from asexual stage parasites – these crescent-shaped gametocytes need to be ingested by *Anopheles* mosquitoes for transmission to occur. In **Figure 1**, the steps of the parasite life cycle involved in human-to-mosquito transmission and the factors believed to influence transmission potential of infected individuals are schematized. Of note, the only naturally-occurring exception to vector-mediated transmission is congenital malaria [9], which is thought to be rare in highly endemic areas and will not be discussed in this document.

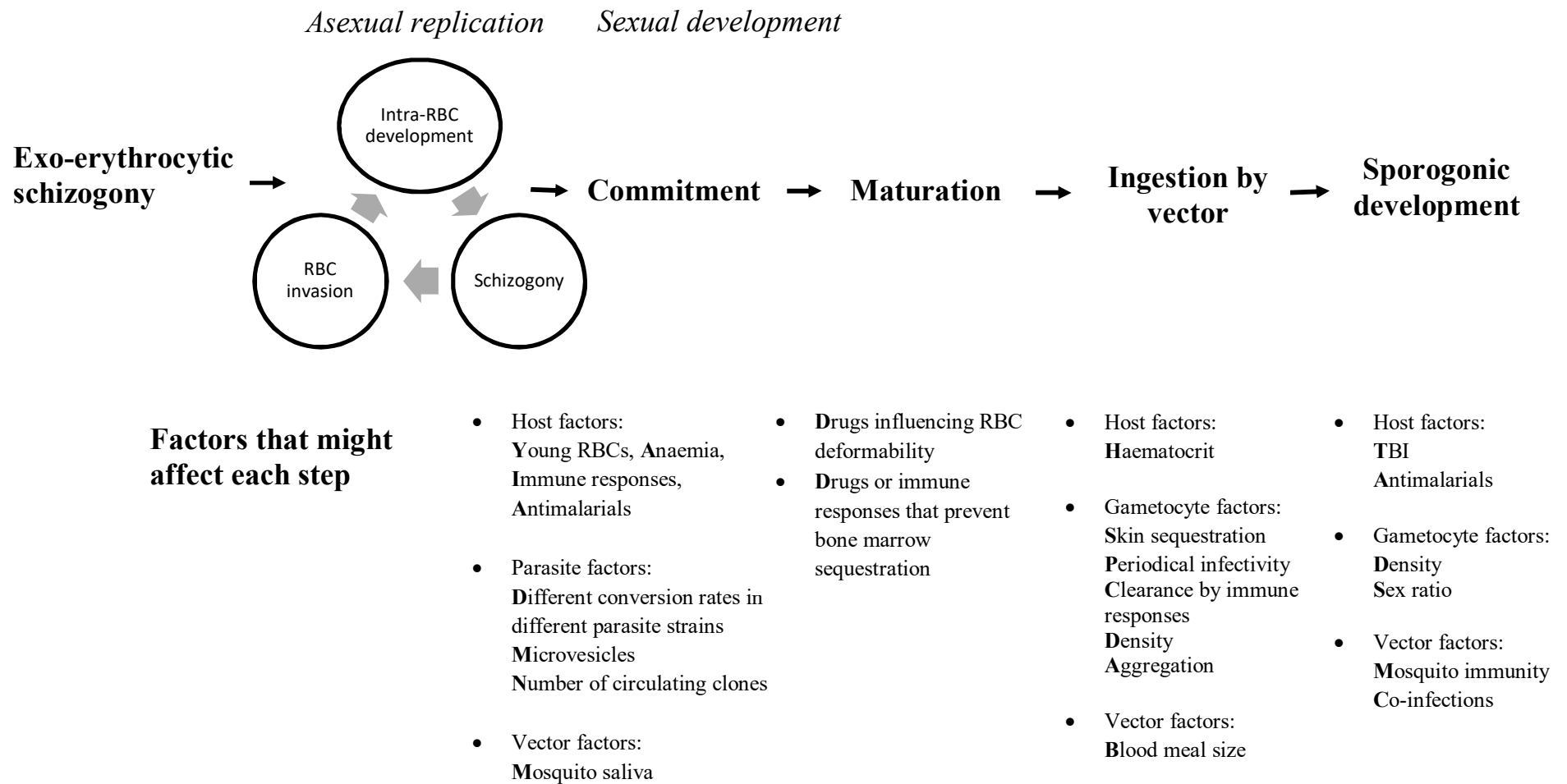
The long process leading to human infectiousness to mosquitoes starts at the moment exo-erythrocytic schizonts in the liver burst and merozoites invade red blood cells: this is the beginning of the blood stage of the malaria infection, responsible for both morbidity and infection propagation. *Falciparum* parasites in these newly infected erythrocytes replicate their genome several times and 48 hours after invasion a new generation of merozoites is produced by schizogony. At each replication cycle, a subset of these erythrocytic schizonts will generate sexually-committed merozoites. Indeed, Bruce and colleagues used monoclonal antibodies that distinguish sexual and asexual blood stage parasites to study the progeny of single *falciparum* schizonts and their *in vitro* work suggested that all merozoites from a single schizont become either replicating asexual stage parasites or gametocytes [10]. Nearly ten years later, another study using red blood cell monolayers and gametocyte-specific antibodies showed that sibling gametocytes derived from a single schizont are all either male or female [11]. The molecular events underlying this commitment to transmission have only recently started to be elucidated. The *ap2-g* gene, a transcription factor epigenetically silenced in asexual stage parasites, is essential to initiate formation of *P. falciparum* gametocytes and thought to control transcription of early gametocyte genes [12]. Other genes that have been linked to the early steps of the sexual development include *Nima-related kinase* (*Pfnek4*) and *P. falciparum* gametocyte development 1 (*Pfgdv1*): the latter was identified by comparative genomic analysis of gametocyte-producing and -deficient parasite lines [13]; the former was shown not to be gametocyte-specific although *Pfnek4*-expressing schizonts generated more gametocytes than schizonts that did not express this gene [14]. The identification of these and other early markers of gametocytogenesis [15] is relevant not only for future studies of gametocyte formation in experimental systems but potentially for field studies of malaria transmission that aim to predict future infectiousness by quantifying levels of sexually-committed parasites.

Although these studies revealed some of the molecular steps of gametocytogenesis (recent findings were reviewed by Josling and Llinás [16]), what determines the “decision” of a parasite or a population of parasites to invest in transmission or to produce replicating parasites remains controversial. In the late 1970s, Carter and Miller [17] showed, by manipulating *falciparum* parasites cultures, that the microenvironment (i.e. human blood) to which malaria parasites are exposed

influences the commitment rate, also called conversion rate, of asexual to sexual stage parasites. Indeed, evidence [18] accumulated over the last three decades suggests that changes in conversion rates might be an adaptive response to signs of stress or to the effects of stress on the parasite population. Stress is often linked to changes in haematological parameters [19] including age of red blood cells and anaemia and to competition for host resources (number of circulating falciparum clones). Factors directly related to parasite clearance, such as human immunity [20] and antimalarials [21-23], have also been associated with enhanced or decreased commitment to gametocytogenesis. Interestingly, microvesicles, thought to be a means of communication between parasites circulating in the same human host, influence gametocytogenesis [24, 25] and could be responsible for one of the mechanisms that control transmission investment. To my knowledge, the only non-stress-related factor that has been postulated to influence commitment to gametocytogenesis is exposure to saliva of uninfected *Anopheles* mosquitoes at the beginning of the wet season in areas with seasonal transmission [26] – such mechanism could explain the observation that in some endemic areas gametocyte prevalence is higher at beginning versus peak of wet seasons [27]. Despite the long list of factors believed to modulate malaria parasite conversion rates, no study has attempted to quantify the contribution of these different stress signals to the establishment of gametocyte populations and for all we know, for an individual parasite commitment to sexual development seems to be the outcome of a stochastic process.

Commitment to gametocytogenesis does not fully determine the contribution of an asexual stage parasite to future transmission. The sex ratio of circulating gametocytes is another key factor that influences the capacity of human hosts to infect mosquitoes [28]. As with commitment, sexual determination in gametocytes remains a mystery and although animal model studies have linked host factors to changes in gametocyte sex ratio during the course of malaria infections [19, 29, 30], the mechanism behind this plasticity continue to elude us.

Figure 1. Factors that influence the transmission of malaria parasites from humans to mosquitoes. Abbreviations: RBC, red blood cell; TBI, transmission blocking immunity.



Red blood cell invasion by sexually-committed merozoites is followed by a maturation period that lasts approximately one week [31] and involves five morphologic stages [32]. Gametocytes in the initial stages of this maturation process (stages II – IV) are not observed in the peripheral circulation, although stage III – IV specific transcripts have been detected in the peripheral blood [33]. In a field study in The Gambia, Smalley and colleagues biopsied children with falciparum infections and observed that immature gametocytes accumulate in the bone marrow [34]. A recent autopsy study of children with fatal malaria [35] and an epidemiological study of children with severe anaemia that included bone marrow biopsy [33] confirmed this observation that young gametocytes accumulate in the bone marrow, more specifically in the extravascular compartment of this organ [36]. Changes in red blood cell membrane rigidity [37], rather than cytoadherence [38], have been implicated in both accumulation of immature gametocytes in the bone marrow and migration of mature gametocytes back to peripheral circulation.

After stage V gametocytes leave maturation sites, our knowledge of what happens with these parasites in the blood is limited. Evidence from malariatherapy studies [39] suggests that stage V gametocytes are not necessarily infectious when they enter circulation and few more days might be necessary for these parasites to fully mature. Furthermore, it is a well-known fact that gametocytes represent only a small proportion of all circulating parasites [40]. This could be explained by at least two mechanisms: low commitment rate (estimated [31] to be ~1%); and the 8-to-12 day lag between commitment and circulation of mature gametocytes means that, if exponential growth of parasites persists during this period, the asexual parasite population when a cohort of gametocytes enters circulation is up to 10^5 times larger than the asexual parasite population at the time of commitment of the same gametocyte cohort (10 represents the approximate effective parasite multiplication in each cycle, as estimate by controlled human malaria infections [41]).

Low gametocytes densities during infections contrast with the success of falciparum parasites in sustaining transmission. Several strategies are believed, with varying supporting evidence, to facilitate the ingestion of gametocytes by malaria vectors: circadian changes in gametocyte infectiousness (also known as Hawking phenomenon named after Frank Hawking), gametocyte aggregation in peripheral blood, and

gametocyte sequestration in skin capillaries. In 1971, Hawking and colleagues [32] observed that exflagellation activity of *P. falciparum* parasites had periodicity of 48 hours, and more recently a study in Tanzania showed that gametocytes densities might peak early at night [42] – however in the same study, the time of peak gametocytaemia did not coincide with the time of peak mosquito biting activity. Evidence of gametocyte aggregation comes from the observation that the distribution of gametocytes in mosquito blood meals does not appear to be random but rather highly heterogeneous, with few mosquitoes ingesting very high quantities of gametocytes; in statistical terms, the number of gametocytes in mosquito blood meals is overdispersed and seems to follow a negative binomial, rather than a Poisson, distribution [43]. To my knowledge, no other studies have been performed that support or refute this finding. Finally, the accumulation of gametocytes in the skin is an obvious and yet difficult-to-test mechanism of maximising the transmission probability of circulating gametocytes. There is limited evidence for this parasite strategy from studies using skin scarification to test for presence of gametocytes in the skin [44]. The fact that mosquito skin feeding experiments, where *Anopheles* mosquitoes feed directly on the skin of volunteers, result in higher proportions of infected mosquitoes compared to membrane feeding assays [45, 46] indicates that preferential accumulation of gametocytes in the skin may occur however it is not possible to rule out that other factors, including changes in parasite fertility linked to venous sampling, are responsible for this observation.

Once inside the mosquito, male gametocytes, which represent ~20% of the total gametocyte population in natural infections [28], exflagellate and generate eight microgametes each. Two factors have been proposed to trigger this process: xanthurenic acid [47] in mosquito midgut and drop in temperature. Each female gametocyte produces only one macrogamete. A zygote is formed from the fertilisation of a macrogamete by a male gamete and differentiates into a motile ookinete. The migration of ookinetes towards the midgut epithelium and the subsequent invasion are followed by a transformation into immotile oocysts. Of note, this process is associated with substantial reductions in parasite numbers, with more than 90% of the initial parasite population (gametocytes ingested) being lost up to the oocyst stage, most of this loss occurring in the gametocyte-to-zygote step (which involves both gametogenesis and fertilisation) (reviewed in [48]). Roughly two weeks after

gametocyte ingestion, sporogonic development is complete and sporozoites developing inside oocysts migrate to salivary glands rendering the mosquito infectious to humans.

Transmission in endemic areas

This highly plastic transmission strategy developed by *falciparum* parasites explains why malaria is so widespread in tropical regions. Indeed, the basic reproductive number or R_0 (the number of secondary infections generated by a single infectious individual in an immunologically naive population) of malaria is estimated to be 115 (median, interquartile range 30 – 815) in endemic areas [49], which is considerably higher compared to infections caused by pathogens with direct transmission mechanism. As with other vector-borne infections, this potential to cause secondary cases depends not only on the number of mosquitoes each individual infects but also on the number of individuals each infected mosquito infects. Knowing that anopheline mosquitoes are abundant in most endemic areas, it is easy to understand why R_0 estimates for malaria reach values as high as ~3,000.

For an infectious individual, the factors that determine the number of mosquito infections he or she will generate can be broadly categorised as factors that influence infectivity, duration of infectious period and exposure to anopheline vectors. The sub-sections discussing these factors were in part based on a review by Stone and colleagues [50], particularly where previous malaria xenodiagnostic studies and studies that assessed age-specific exposure to mosquitoes are described. This review is included in the *Appendix* for reference.

Infectiousness

Logic and intuition suggest that the probability of mosquito infection after a single blood meal is directly linked to total parasite burden in the human host, as asexual parasites generate the sexual stage parasites. However, the time necessary for the maturation of *P. falciparum* gametocytes means that circulating gametocytes densities might only loosely correlate with concomitant asexual parasitaemia. An alternative approach to estimate the infectiousness of infected individuals is to quantify their

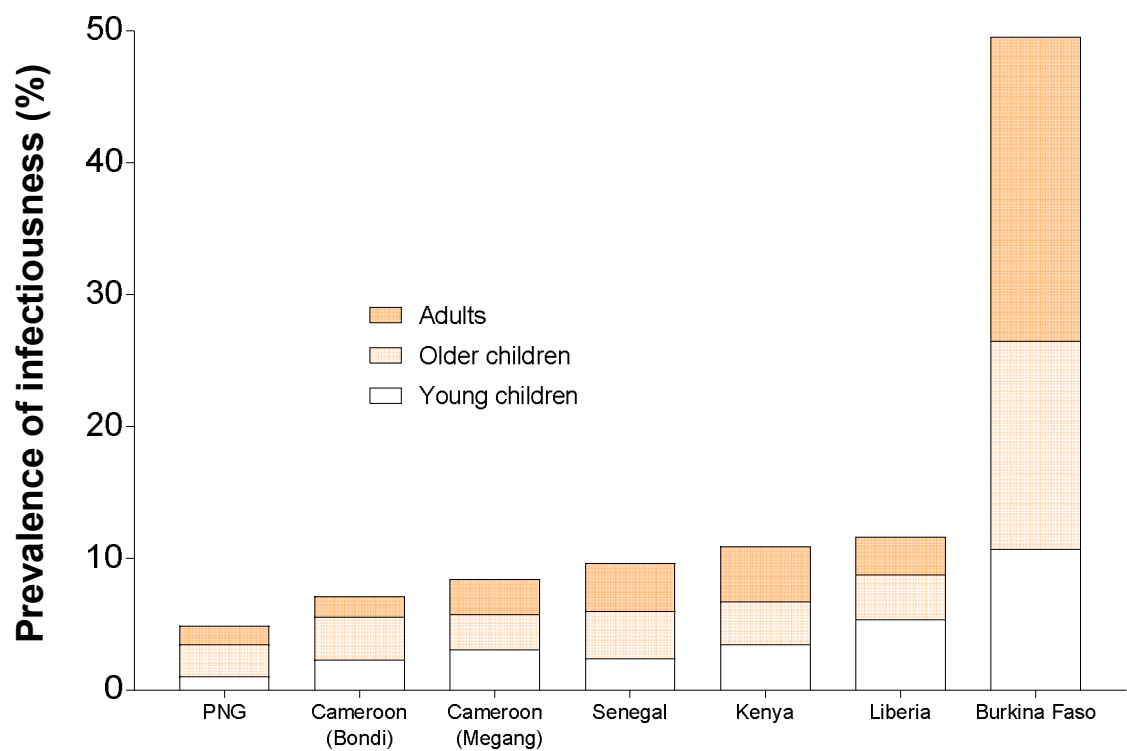
gametocyte densities. Transmission studies where mosquitoes were fed on blood or skin of volunteers suggest a positive correlation between the density of gametocytes in the blood and the risk of mosquito infection [51-53]. Predictions of mosquito infection rates (i.e., proportion of mosquitoes acquiring infection in feeding assays) based solely on gametocyte density data are however complicated by the shape of this relationship, which is sigmoidal, not linear, and seems to be dependent on how we quantify gametocytes in the peripheral blood [52]. The decades-old observations [54, 55] that individuals with submicroscopic densities of gametocytes can infect mosquitoes, and that some individuals with high gametocytaemia fail to produce mosquito infections confirm that gametocyte density, although important, is not the only determinant of host infectivity. Several studies (reviewed in [56]) have shown that some but not all individuals living in endemic areas acquire immunity that reduces infectiousness in mosquito feeding assays. Other host factors, such as genetics might influence gametocyte levels [57, 58] and infectivity [59] as well as mosquito survival after feeding experiments [60].

This multitude of factors modulating the infectiousness of malaria-infected individuals suggests that to determine how many mosquitoes an individual can infect all these different parameters need to be measured. A more direct approach to estimate infectivity is to let female *Anopheles* mosquitoes feed on infected human blood or on the skin of volunteers. This procedure was called xenodiagnostic by the French parasitologist Émile Brumpt, studying Chagas disease, who described this type of assay as “une culture naturelle du parasite chez des hôtes favorables” [61].

Identifying the individuals who drive malaria transmission in endemic areas and quantifying the contributions of children and adults to local epidemiology are the ultimate goals of using these transmission assays. Sixty years ago, the xenodiagnostic method was used in a first attempt to quantify infectiousness at a population level [62]. Changing the focus from individuals to entire populations was a major development. This initial survey was performed in an area with endemic transmission in Liberia; individuals were enrolled in the study regardless of parasite status and mosquitoes were fed on their skin to assess infectivity. Approximately 10% of the population were capable of infecting mosquitoes and although young children were more often infectious, all different age groups contributed similar numbers of infectious

individuals to the local malaria transmission reservoir. After this first study, few other xenodiagnostic surveys were performed [63-67]. In all these surveys, except one in Burkina Faso where the prevalence of infectiousness was 48%, only a small percentage ($< 15\%$) of naturally exposed individuals contributed to local transmission (**Figure 2**). Recent epidemiological studies using molecular methods to detect malaria parasites indicate that even in low transmission areas significant proportions of populations are infected with malaria parasites [68]. The findings of xenodiagnostic surveys suggest that the majority of these infected individuals are, at least temporarily, non-infectious, possibly due to relatively low gametocyte densities.

Figure 2. Xenodiagnostic surveys. Infectiousness (infection of at least one mosquito in feeding experiments) prevalences are presented in the y-axis. Previous assessments of the malaria infectious reservoir are listed in the x-axis [62-67]. The proportions of infectious individuals from different age groups are indicated. Surveys in Burkina Faso and Senegal did not include children aged 5 years or younger and infectiousness prevalences in older children (aged between 5 and 15 years) were used for both age groups. Demographic data from World Population Prospects (United Nations) were used to calculate age-adjusted prevalences of infectiousness.

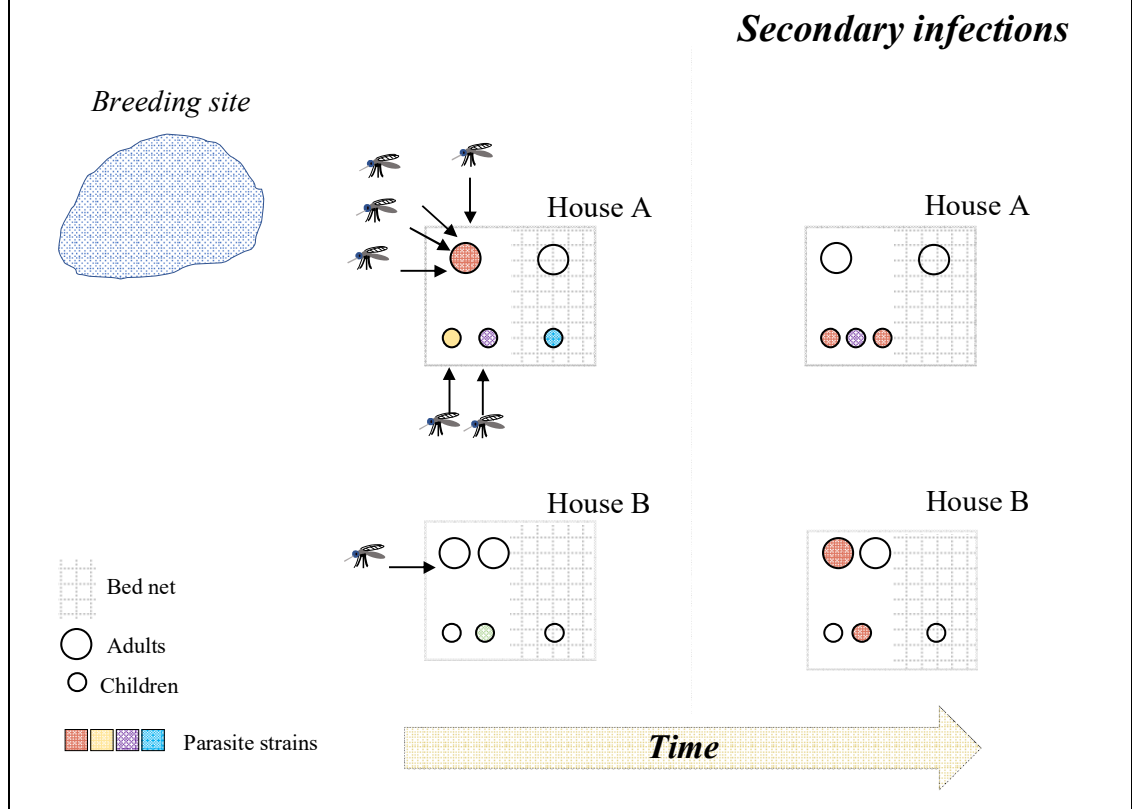


All population-wide malaria xenodiagnostic studies were cross-sectional surveys and consequently were not able to assess duration of infectivity. In endemic areas, children are more likely to develop symptoms that require treatment compared to adults. Since effective antimalarials limit the duration of gametocyte circulation [69] and consequently of infectivity, older children and adults, who acquired immunity that protects against clinical malaria and might remain asymptomatic for several weeks or months, might carry gametocytes for longer periods of time after a new infection is established. Blood stage immunity that clears asexual stage infection might also influence the duration of infectiousness, especially in chronic asymptomatic infections. To determine the range of durations of malaria infectiousness in naturally acquired infections, longitudinal studies enrolling randomly selected individuals and repeatedly assessing infectiousness at regular intervals would be necessary.

Transmission opportunities

The number of mosquito bites a malaria-infected individual receives has a direct impact on his or her contribution to local transmission (**Box 1**). This number depends on local vector density, and on the degree of anthropophily (proportion of all mosquito blood meals originating from humans) of mosquitoes: while *Anopheles gambiae sensu stricto* and *Anopheles funestus* are predominantly anthropophylic, another important malaria vector species, *Anopheles arabiensis*, presents some degree of zoophily [70]. These factors determine the average exposure to malaria vectors in a community; however, different individuals of the same community are not equally exposed to mosquitoes and several studies [71-73] indicate that there is substantial variation in human attractiveness to *Anopheles* mosquitoes.

Box 1. Exposure to *Anopheles* mosquitoes determines contribution to local transmission. In the figure below, malaria transmission in two hypothetical households is presented. Household A is located near a mosquito breeding site and individuals living in this house are exposed to higher vector densities compared to individuals in household B. Adults are represented by large circles and are assumed to be more attractive to mosquitoes than children (smaller circles). Individuals sleeping under bed nets were placed in hatched areas. Relative mosquito exposure is represented by the number of mosquitoes linked to each resident of these houses. Coloured circles correspond to infected individuals, and the colours of the circles in the right panel of the figure represent the source of the infections: for example, the infected adult in household A was the source of most human infections detectable few weeks later.



Studies that quantified age-specific exposure to mosquitoes by direct observation or by ABO typing of blood meals indicate that adults are more attractive than children to *Anopheles* mosquitoes [74-77]. This age pattern in vector exposure seems to be related to differences in body weight and surface area [77]. Additional factors that may influence host attractiveness include body temperature and chemical cues [78-80]. Behaviour that prevents mosquitoes from feeding after alighting on the skin also seems to change with age [74]. The observation that individuals with gametocytes might be more attractive to mosquitoes [81] suggest that the parasite might modulate host attractiveness to vectors to maximise its transmission. Of note, a recent study identified a chemical compound produced by *P. falciparum* parasites that could mediate enhanced attractiveness of malaria-infected individuals to mosquitoes [82].

Human behaviour, including use of bed nets, also influences how often individuals are exposed to mosquitoes. In areas where nets are widely used, age-specific bed net coverage is highest among young children [83-86]. A reduction in availability of one group could divert mosquitoes to other demographic groups: in The Gambia, children were protected against malaria after insecticide-treated bed nets (ITN) were introduced; this protection was at least partially related to the diversion of mosquito bites to animals or adults [87]. Other behavioural practices, such as sleeping times and activities performed indoors or outdoors at night, influence availability to different mosquito populations. If children sleep earlier than their parents, they might be protected by bed nets for longer periods of time every night. On the other hand, if adults stay outdoors more often than children, they might be an important source of outdoor malaria transmission, even if they are less infectious. How different behavioural factors influence transmission will depend on local vector populations.

Preventing human-to-mosquito transmission of falciparum parasites

Malaria transmission events can be prevented by reducing the infectiousness of human hosts or by minimising the number of host-vector encounters. While the latter can be achieved by bed net use and indoor residual spraying of insecticides, infectivity reduction relies on drugs that target different steps of the malaria transmission process (**Figure 1**). This section will focus on how antimalarials influence human infectivity.

The clearance of asexual stage parasites by antimalarials limits production of gametocytes and consequently infectivity duration. Artemisinin-based combination therapies (ACTs), in addition to effectively clearing asexual parasites [88], also have activity against immature gametocytes [89], which explains the lower post-treatment infectivity associated with these drug combinations [90]. Mature gametocytes however seem to be only mildly affected by ACTs [89] and this has been associated with transmission after treatment initiation [91]. In areas approaching malaria elimination, where few additional infected mosquitoes might have a significant impact on the persistence of endemic transmission, antimalarials should completely sterilise parasites shortly after drug administration. Primaquine can accelerate clearance of circulating stage V gametocytes and has been recommended to reduce infectiousness of falciparum infections in combination with first-line antimalarials. Questions remain on which primaquine doses should be used in combination with different ACTs [92] and how primaquine should be deployed (e.g. mass drug administration campaigns). A better understanding of the malaria infectious reservoir is essential to inform chemotherapy-based approaches to interrupt transmission.

Summary

Human-to-mosquito malaria transmission is an intricate and robust process that allows falciparum parasites to propagate despite the wide range of host (peripheral blood) environments. At the community level, identifying individuals who contribute to malaria transmission requires data on human infectiousness to mosquitoes and estimates of mosquito exposure. Indeed, infectious individuals who are frequently exposed to anopheline vectors constitute the local human infectious reservoir of malaria. The aims of the work presented here were to quantify the infectiousness of individuals living in falciparum malaria endemic areas (Chapter 3), their exposure to *Anopheles* mosquitoes (Chapter 4) and the benefits of using primaquine as an infectiousness-reducing intervention (Chapter 5).

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Chapter 2, Objectives

The following chapters describe epidemiological (**Chapter 3**), entomological (**Chapter 4**) and clinical (**Chapter 5**) studies on falciparum malaria transmission. These different approaches were used to address interconnected questions related to the spread of malaria parasites in natural settings.

Rationale

Heterogeneity pervades various aspects of *P. falciparum* epidemiology – this now widely held view is supported by observations that few individuals suffer most clinical malaria episodes [1] and that relatively small areas concentrate most malaria burden of a community [2]. While these findings concern infection risk and disease susceptibility, human malaria transmission potential, the capacity of an infected individual to generate secondary mosquito infections, is also thought to be variable, although evidence supporting this comes from disconnected studies that determined either human infectiousness or attractiveness to mosquitoes (see **Chapter 1**). A realistic quantification of the variability in transmission potential would be informative for control programmes and allow optimal implementation of strategies that target the transmission process (e.g. administration of gametocytocidal drugs).

Objective

The research projects presented here had as general objective a better understanding of falciparum transmission at a community level, which could inform the deployment of infectiousness-reducing tools such as primaquine. These studies were complementary to each other in that quantifying the two parameters that influence malaria transmission from humans to mosquitoes (infectiousness and actual exposure to malaria vectors) required different methodologies.

Specific objectives

Specific objectives are either related to more comprehensive descriptions of human transmission potential (first three objectives) or the direct evaluation of a drug that could interrupt infectiousness (fourth objective):

- To determine the infectiousness of individuals living in malaria endemic areas and how it relates to standard and molecular parasite diagnostics
- To estimate how often individuals are exposed to *Anopheles* mosquitoes and how vector exposure varies between individuals and over time
- To quantify age-specific contributions to malaria transmission based on infectiousness and vector exposure estimates
- To assess the effect of primaquine on the infectiousness of individuals infected with falciparum parasites.

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Chapter 3, The human infectious reservoir for *Plasmodium falciparum* malaria in areas of differing transmission intensity

This chapter describes the results of xenodiagnostic surveys performed in two villages in Burkina Faso. This work, together with data from two other study sites (Mbita and Kilifi, both in Kenya), was submitted for publication. The version presented here is a revised version after initial comments from reviewers. In addition to the manuscript and the supplementary material, two new figures are included at the end of this chapter.

In October 2017, this manuscript was published in *Nature Communications* (<https://doi.org/10.1038/s41467-017-01270-4>).



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SECTION A – Student Details

Student	Bronner Pamplona Augusto Gonçalves
Principal Supervisor	Chris Drakeley
Thesis Title	The human infectious reservoir of falciparum malaria

If the Research Paper has previously been published please complete Section B, if not please move to Section C

SECTION B – Paper already published

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SECTION C – Prepared for publication, but not yet published

Where is the work intended to be published?	Nature Communications
Please list the paper's authors in the intended authorship order:	Bronner P. Gonçalves [#] , Melissa C. Kapulu [#] , Patrick Sawa, Wamdaogo M. Guelbéogo, Alfred B. Tiono, Lynn Grignard, Will Stone, Joel Hellewell, Kjerstin Lanke, Guido J. H. Bastiaens, John Bradley, Issa Nébié, Joyce M. Ngoi, Robin Oriango, Dora Mkabili, Maureen Nyaurah, Janet Midega, Dyann F. Wirth, Kevin Marsh, Thomas S. Churcher, Philip Bejon, Sodiomon B. Sirima, Chris Drakeley, Teun Bousema
Stage of publication	Under review (a revised version of the manuscript is included in this chapter)

SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper	I wrote the first draft, with MK, TB and CD. I performed the statistical analyses included in this version of the manuscript.
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Title page

Title: The human infectious reservoir for *Plasmodium falciparum* malaria in areas of differing transmission intensity

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Abstract

Detailed understanding of the human infectious reservoir is essential to rationally target malaria transmission-reducing interventions. We report on the first multi-region study to assess population-wide malaria transmission potential based on 1,209 mosquito feeding assays in endemic areas in Burkina Faso and Kenya. Overall, 39 individuals were infectious; age-standardised prevalences of infectiousness in the study sites ranged from 0.9 to 6.4%, and the percentage of infected mosquitoes ranged from 0.05% (4/7,716) to 1.6% (121/7,749) and correlated positively with transmission intensity. In the high endemicity setting, most infectious individuals were identified by research-grade microscopy (92.6%; 25/27), whilst 1 of 3 infectious individuals in the lowest endemicity setting was detected by molecular techniques alone. Exposure to malaria vectors was estimated by genetically matching blood from 1,094 wild-caught bloodfed mosquitoes with that from humans resident in the same houses. Although adults transmitted fewer parasites to mosquitoes than children, they received more mosquito bites, balancing their contribution to the infectious reservoir.

Introduction

Heterogeneity in the transmission potential of individual hosts is a common feature of many infectious diseases, and the identification of individuals who disproportionately contribute to onward transmission has attracted much attention [1, 2]. For vector-borne infections, quantifying the contribution of individual humans to transmission (the infectious reservoir) requires the estimation of host infectivity with the pathogen and assessment of the effective contact rate, the frequency with which individual hosts are sampled by the vector population. Malaria due to *Plasmodium falciparum* remains a major cause of morbidity and mortality worldwide, despite marked recent declines in disease burden [3, 4]. Initiatives to further reduce the burden of malaria, as well as efforts to contain the spread of artemisinin resistant malaria parasites in Southeast Asia [5], require a thorough understanding of the human infectious reservoir for malaria, which would allow interventions to be targeted to individuals who are most important for the transmission of infection to mosquitoes. The need to resolve uncertainties in identifying these individuals is further necessitated by accumulating evidence that a considerable proportion of those infected have very low parasite densities, only detectable by sensitive molecular assays. This need is particularly acute in low malaria transmission settings and areas threatened by artemisinin resistance [6, 7].

Few epidemiological studies have assessed malaria infectiousness at a population level by directly quantifying human infectivity using mosquito feeding experiments [8-12]. In these experiments, malaria vectors are fed directly on the skin or on blood through a membrane and later dissected for parasite development assessment (e.g., oocyst detection). Only one of these studies, conducted in an area of intense malaria transmission in Burkina Faso, concurrently quantified asexual stage parasites and the transmissible sexual stage parasites (gametocytes) by molecular assays and concluded that up to 17% of mosquito infections are caused by submicroscopic parasite carriage in humans [12]. In contrast, in Cambodia a hospital-based study involving uncomplicated malaria cases reported much lower infectivity of submicroscopic infections [13]. The findings of these studies highlight the need for more extensive assessments of infectivity to mosquitoes across a range of endemicities. Such xenodiagnostic surveys select individuals regardless of parasite carriage, incorporate concurrent assessments of parasite and gametocyte densities by molecular assays,

which could provide insights into the mechanisms responsible for the variation in human-to-mosquito infectiousness, and take into account that individuals are not equally bitten by mosquitoes and consequently have different numbers of opportunities to transmit [14]. Both infectiousness and exposure to mosquito bites are needed to be able to quantify an individual's contribution to malaria transmission.

In this study, we determined the proportions of mosquito infections originating from different demographic groups in areas with low, moderate and high malaria transmission intensity. For this, we integrated infectivity data from xenodiagnostic surveys, molecular parasite quantification and data on actual exposure to malaria vectors, which were generated by blood meal analysis of wild-caught bloodfed mosquitoes in study sites in East and West Africa.

Results

Xenodiagnostic surveys were performed in four areas selected to represent three different malaria endemicities (**Table 1**): in Burkina Faso, the villages of Laye and Balonghin, sampled during the low intensity season (from here on 'dry season') of 2013 and the peak intensity season (from here on 'wet season') of 2014 respectively, are both characterised by intense seasonal transmission; malaria transmission in Mbita, Kenya, is seasonal with moderate intensity; in Kilifi, also located in Kenya, low transmission occurs throughout the year but is higher during the wet season months. Membrane feeding experiments were used to quantify infectivity of study participants to locally reared *Anopheles gambiae* mosquitoes [12, 15].

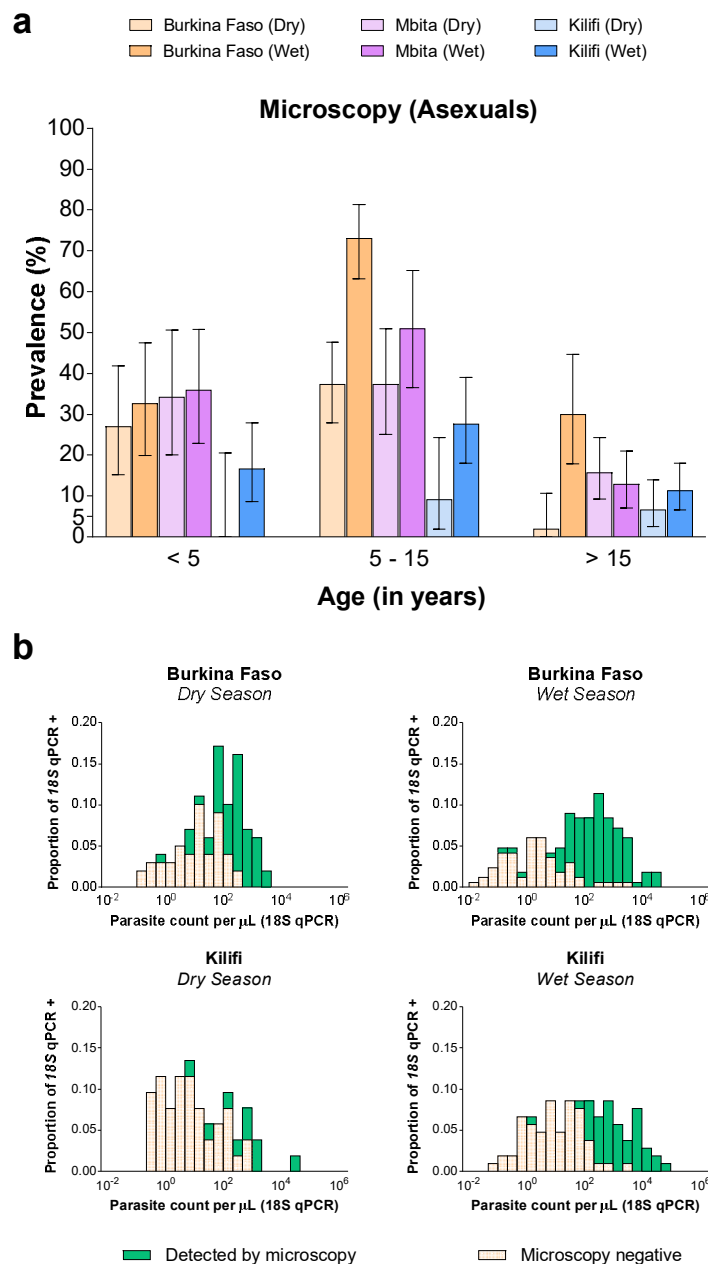
Table 1. Xenodiagnostic surveys. In Mbita, 142 individuals participated in both dry and wet season surveys.

	Laye (Burkina Faso)	Balonghin (Burkina Faso)	Mbita (Kenya)	Mbita (Kenya)	Kilifi (Kenya)	Kilifi (Kenya)
Transmission						
<i>Intensity</i>	High		Moderate		Low	
<i>Season</i>	Dry	Wet	Dry	Wet	Dry	Wet
Number of Participants	200	200	202	202	139 ^a	274
Start Date	May – 2013	Oct – 2014	Feb – 2014	May – 2014	Jan – 2014	May – 2014
End Date	Jun – 2013	Nov – 2014	Apr – 2014	Jul – 2014	Apr – 2014	Dec – 2014
	<i>N (%)</i>	<i>N (%)</i>	<i>N (%)</i>	<i>N (%)</i>	<i>N (%)</i>	<i>N (%)</i>
Age categories (in years)						
< 5	50 (25.0)	49 (24.6)	41 (20.3)	50 (24.7)	16 (11.5)	66 (24.1)
5 - 15	100 (50.0)	100 (50.2)	59 (29.2)	51 (25.2)	33 (23.7)	76 (27.7)
> 15	50 (25.0)	50 (25.1)	102 (50.5)	101 (50.0)	90 (64.7)	132 (48.2)
Gender						
<i>Male</i>	98 (49.7)	114 (57.3)	69 (34.2)	86 (42.6)	43 (30.9)	108 (39.4)
<i>Female</i>	99 (50.2)	85 (42.7)	133 (65.8)	116 (57.4)	96 (69.1)	166 (60.6)

^a35 participants were recruited in January – February 2015

A total of 1,075 individuals were recruited regardless of their parasitological status, including 142 who participated in both dry and wet season surveys in Mbita (**Table 1**), with a total of 1,216 parasitological observations. As expected, infection with *P. falciparum* was more prevalent in high (Burkina Faso) versus moderate (Mbita) and low (Kilifi) transmission settings (**Figure 1a**). While parasite positivity was higher during wet versus dry season in Burkina Faso (83.8% [166/198] and 50.5% [100/198] by *18S* qPCR, respectively), in Kilifi, *P. falciparum* prevalence was similar in both seasons (37.4% [52/139] versus 38.3% [105/274] by *18S* qPCR during dry and rainy seasons, respectively). Molecular detection and quantification of malaria parasites failed for samples collected in Mbita due to local electricity issues resulting in an unknown number and duration of freeze-thaw cycles. Based on microscopy results, the prevalences of asexual stage parasites in the two Mbita surveys were also similar (25.7% [52/202] versus 28.2% [57/202] during dry and wet seasons, respectively). In all sites, children aged between 5 and 15 years had higher parasite prevalence than individuals older than 15 years (**Figure 1a**). The detectability of infections by microscopy was associated with qPCR parasite density (**Figure S1**); the median (interquartile range [IQR]) parasite densities estimated by *18S* qPCR in microscopically subpatent and patent infections were 4.9 (1.0 – 32.6) and 387.3 (95.1 – 1,244.5) parasites per μL , respectively. In Balonghin, Laye, Kilifi wet season and Kilifi dry season the percentages of infections that were detectable by *18S* qPCR but negative by microscopy (i.e. submicroscopic infections) were 37.9, 49.5, 58.1 and 82.7%, respectively (**Figure 1b**). The distributions of parasite densities estimated by *18S* qPCR suggest that the tails of undetectable infections (infections with densities below 0.01 parasites per μL) are negligible for all settings except in Kilifi dry season and may be interpreted as evidence that our assays detected the majority of malaria infections [6]. *18S* qPCR parasite densities were lower in adults compared to children living in the same study area ($P < 0.001$).

Figure 1. Age-specific asexual stage parasites prevalence by microscopy (a) and distributions of parasite densities (b) by study site. In **a**, 95% exact binomial confidence intervals are presented for microscopy-based parasite prevalence estimates. In **b**, parasite densities quantified by DNA-based *18S* qPCR are presented (\log_{10} scale) for both patent and subpatent infections. This panel only includes *18S* qPCR-positive samples (100, 166, 52 and 105 in Burkina Faso dry and wet season surveys, and Kilifi dry and wet season surveys, respectively).



A small percentage (6.2% [75/1213]) of samples had microscopically detectable levels of gametocytes. As expected, *Pfs25* mRNA quantitative nucleic acid sequence-based amplification (QT-NASBA) identified gametocytes in a much larger percentage of the study population (353/809 [43.6%] participants in Burkina Faso and Kilifi surveys). Overall, 76.6% (324/423) of all *18S* qPCR parasite positive individuals were also *Pfs25* mRNA QT-NASBA gametocyte positive. *Pfs25* mRNA QT-NASBA identified more gametocyte carriers in the wet compared to the dry season in both high-endemic Burkina Faso (75.2% [149/198] versus 49.5% [98/198]) and low-endemic Kilifi (29.6% [81/274] versus 18.0% [25/139]). Similarly, gametocyte densities estimated by *Pfs25* mRNA QT-NASBA were higher during wet compared to dry season in Kilifi and in Burkina Faso ($P < 0.001$). As with asexual stage parasites, schoolchildren were more frequently gametocytaemic than adults (63.0 versus 34.0% in dry-season Laye [$P = 0.001$], 86.9 versus 78.0% in wet-season Balonghin [$P = 0.16$], and 44.7 versus 26.5% in Kilifi during the wet season [$P = 0.007$], respectively), except in Kilifi during the dry season (12.1 versus 21.1%, respectively [$P = 0.26$]).

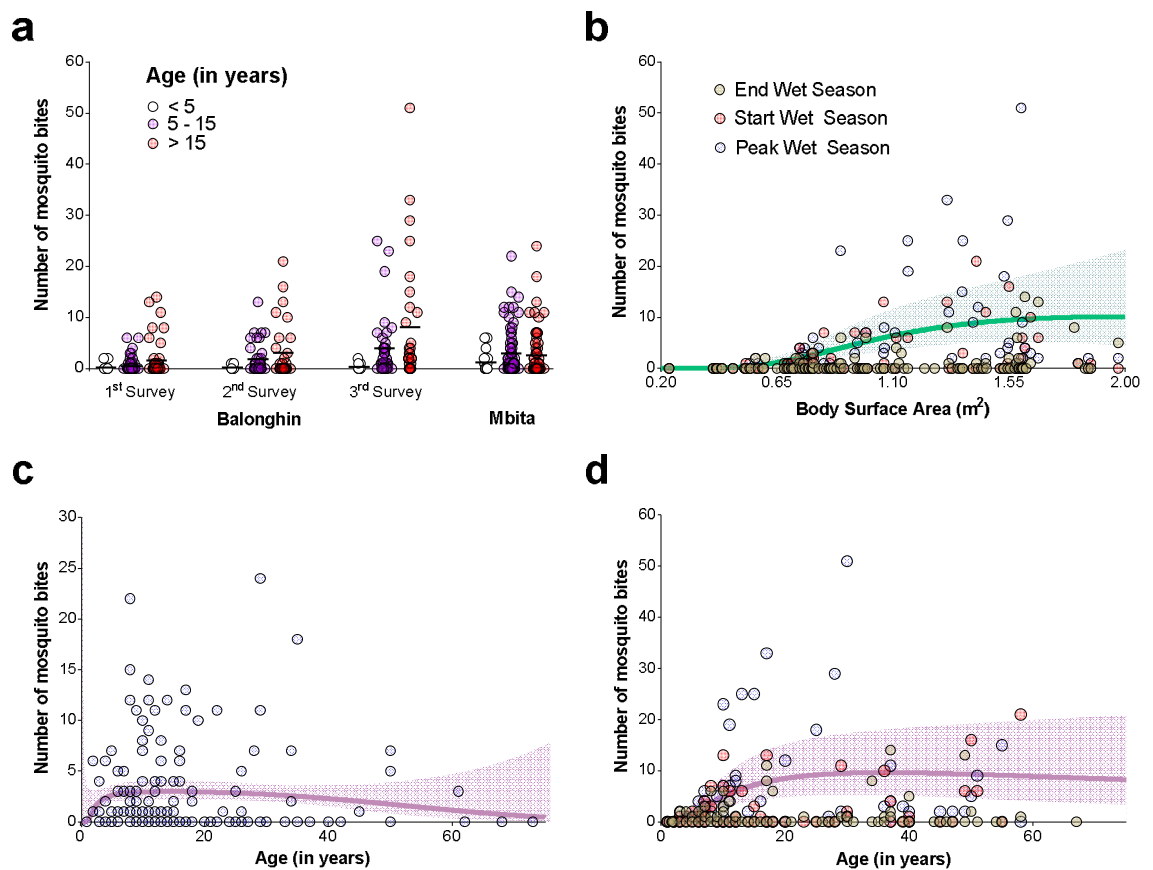
Exposure to mosquitoes is dependent on age and setting

To generate realistic quantification of the transmission potential of individuals, we measured exposure to anopheline vectors to determine the likely frequency with which potentially infectious individuals are sampled by mosquitoes. We analysed a total of 1,874 blood meals from fed mosquitoes (1,066 from Balonghin and 808 from Mbita) collected in households in the study villages to estimate human host age-specific exposure to malaria vectors. Sampling bloodfed mosquitoes in the low endemic area in Kilifi was abandoned when 2 months of sampling yielded only 31 bloodfed mosquitoes, which was insufficient for meaningful assessments of mosquito exposure by age groups. Molecular typing of blood meals to identify the human blood source using a short tandem repeat multiplex PCR assay was successfully performed for 966 and 689 mosquitoes collected in Burkina Faso and Kenya, respectively. Blood was taken from 99.2% (126/127) and 86.7% (163/188) of all household members in Balonghin and in Mbita, respectively, to link to mosquito blood meals.

666 and 428 mosquito blood meals were matched to single individuals living in the same household where they were collected in Balonghin and Mbita, respectively. Since

the association between mosquito exposure and age varied with study site ($P = 0.001$, **Figure 2a, c and d**), we performed separate analyses for the two sites. In Burkina Faso, adults (incidence rate ratio [IRR] 20.9 95% confidence interval [CI] 7.7 – 57.4) and children aged 5 to 15 years (IRR 7.7 95% CI 2.9 – 20.8) were more often bitten by *Anopheles* mosquitoes than younger children (< 5 years). In Mbita, although children aged < 5 years received relatively fewer bites compared to the other age groups, there was no statistically significant difference: IRR 2.3 (95% CI 0.8 – 6.2) for children aged 5 – 15 years versus younger children (reference group) and IRR 2.1 (95% CI 0.7 – 5.7) for adults relative to children aged < 5 years. In Balonghin, weight and height data were collected and used to estimate body surface area. In this setting, higher body surface area was correlated with age and was associated with more frequent mosquito exposure (**Figure 2b**).

Figure 2. Mosquito exposure by age and body surface area. In **a**, the number of mosquito bites for each age group and survey is presented; each circle represents a study participant, and the mean number of mosquito bites per individual by age group is represented by horizontal black lines. In **b-d**, fractional polynomials were used to determine the models that best describe the relationships between mosquito bites and age in Mbita (**c**) and Balonghin (**d**) and mosquito bites and body surface area calculated using Dubois equation (**b**, data from Balonghin). The lines in panels **b-d** represent predicted numbers of mosquito bites per individual based on selected models (see *Methods* section). The green (**b**) and purple (**c, d**) areas represent 95% confidence intervals.



In both study sites, a non-negligible percentage of mosquito blood meals (15.8% [153/966] in Balonghin and 12.6% [87/689] in Mbita) had multiple human DNA sources, indicative of mosquitoes feeding on two or more individuals the night before collection. Since it was not possible to unequivocally determine the sources of human alleles in these blood meals, mosquitoes with multiple human blood sources were not included in the statistical analyses. We assessed whether these mosquitoes with unknown multiple blood meal sources may have affected our estimations of age-specific biting patterns. For this, we identified alleles (N=125) that were unique to single individuals at the household level based on data from Balonghin, Burkina Faso. Fourteen of these unique alleles were in children aged < 5 years living in households where at least one multiply fed mosquito was collected. None of these unique alleles in young children were present in multiple-source blood meals. To further assess the sensitivity of our outcomes to biting preferences of mosquitoes with multiple human blood sources, we re-analysed our data assuming 1) that multiple-source meals were single blood meals equally allocated to study participants living in the household where they were collected, or 2) that individuals who had the lowest genetic distances to the genotypes present in these meals were blood sources: under these different assumptions, adults were still seven to eleven times more likely to be bitten by mosquitoes than young children in Balonghin (see *Supplementary Information*).

Infectivity to mosquitoes by setting, season and age

Overall, 1,209 mosquito feeding assays were performed to assess infectivity of study participants; 39/1,209 (3.2%) individuals infected at least one mosquito (**Table 2**). No mosquitoes (0/3,046) became infected in feeding experiments performed in Kilifi during the dry season. In the other surveys, the percentages of mosquitoes that became infected ranged from 0.05% (4/7,716) to 1.6% (121/7,749) and was highest in Balonghin. Children aged 5 to 15 years had similar infectivity compared to younger children (odds ratio [OR] 1.02, 95% CI 0.48 – 2.20, P = 0.95) and were more often infectious to mosquitoes than adults, albeit not statistically significantly (OR 2.07, 95% CI 0.88 – 4.91, P = 0.10; **Table 3** and **Figure S2**). In Burkina Faso, where the number of infectious individuals was highest, nearly all infectious individuals (25/27, 92.6%) had falciparum parasites, asexual or sexual stages, detected by intensive, research quality, microscopy effectively screening 200 fields, which is equivalent to

~4,000 white blood cells (**Figure S3**). In Kilifi and Mbita, 2/3 (66.7%) and 7/9 (77.8%) infectious individuals, respectively, had microscopically detectable falciparum parasites. 6/9 infectious individuals in Mbita surveys had patent *Plasmodium malariae* infections; in Laye, 1/14 infectious individual only had patent *P. malariae* infection (see *Supplementary Information, Table S1*). Among all 31/39 infectious individuals without evidence of co-infection with non-falciparum malaria parasites, *P. falciparum* gametocytes were detected by microscopy in 61.3% (19/31) and by *Pfs25* mRNA QT-NASBA in 100.0% (28/28). Gametocyte densities by *Pfs25* mRNA QT-NASBA were positively associated with parasite densities by *18S* qPCR (**Figure 3a**; Spearman's rank correlation coefficient for samples with positive *18S* qPCR result 0.35, $P < 0.001$) and positively associated with mosquito infection rates (**Figure 3b**). Mosquito infections were mostly observed when feeding on blood containing estimated gametocyte densities ≥ 10 gametocytes per μL (25/28 infectious feeds). The infection burden in mosquitoes (oocyst density) was positively associated with the proportion of infected mosquitoes (**Figure 3c**), as previously reported for experiments with cultured gametocytes [16, 17] and natural infections with *Plasmodium vivax* [18]. High oocyst counts (10 or more oocysts per midgut) were observed in a subset (29/231) of infected mosquitoes in Burkina Faso while in Kilifi all infected mosquitoes had oocyst counts below 5.

Table 2. Membrane feeding assays.

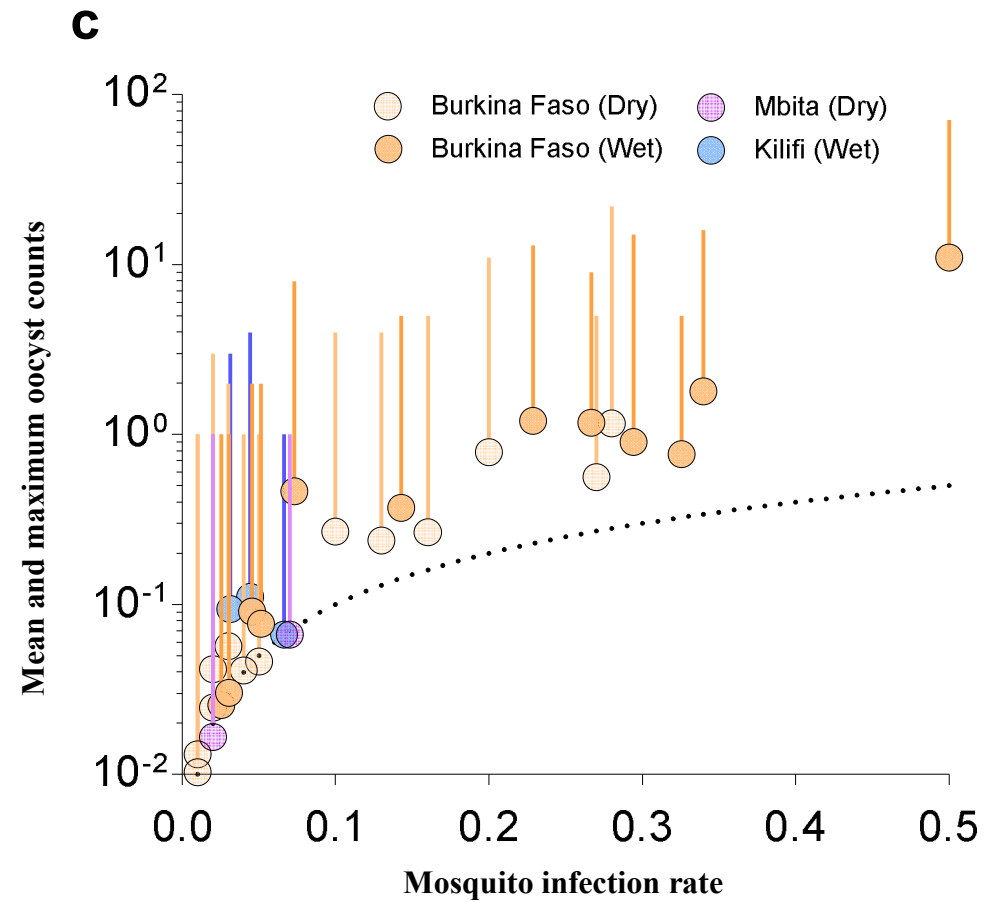
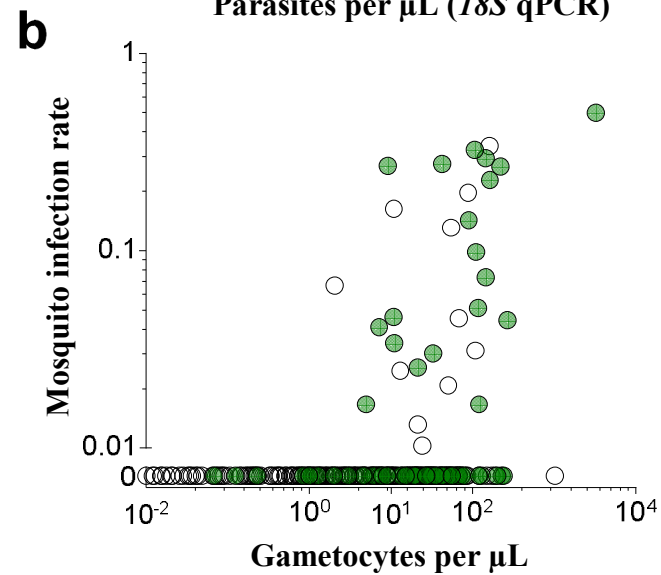
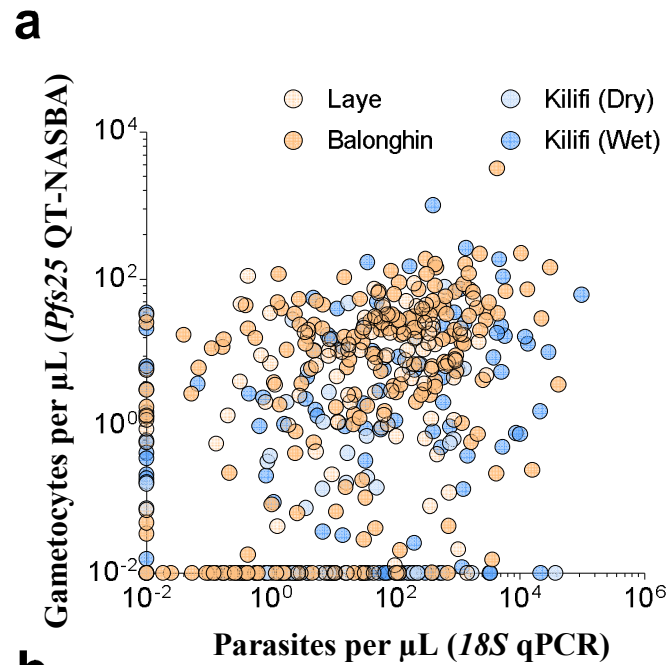
Study Site	Season	Number of participants	Number of mosquitoes dissected	Number of infectious individuals	Number of infected mosquitoes	Number of mosquitoes dissected per participant ^a	Proportion of infected mosquitoes per participant ^b	Median oocyst count (range)
<i>Burkina Faso</i>	Dry	198	17,231	14	110	91 (79 – 97)	0.04 (0.01 – 0.27)	2 (1 – 22)
	Wet	196	7,749	13	121	41 (35 – 46)	0.23 (0.02 – 0.50)	3 (1 – 71)
<i>Mbita, Kenya</i>	Dry	202	7,071	7	28	30 (28 – 49)	0.02 (0.02 – 0.57)	4 (1 – 197)
	Wet	200	6,842	2	5	30 (27 – 46)	0.07, 0.8	2 (1 – 4)
<i>Kilifi, Kenya</i>	Dry	139	3,046	0	0	17 (11 – 31)	-	-
	Wet	274	7,716	3	4	28 (19 – 35)	0.04 (0.03 – 0.07)	2 (1 – 4)

^aMedian (interquartile range); ^bMedian (range), only data from infectious individuals included.

Table 3. Age-specific contributions to local malaria transmission. For the high (Burkina Faso) and moderate (Mbita, Kenya) transmission settings, local age-specific exposure to mosquitoes was quantified and used to estimate the contribution of different age groups to the pool of infected mosquitoes. For Kilifi estimates, age-specific mosquito exposure determined in Mbita was used. Estimates in this table include *P. falciparum* mono-infections and mixed or non-falciparum infections.

		Prevalence of infectiousness % (number of infectious individuals/total number of participants)			% of infected mosquitoes (number of infected mosquitoes/number of dissected mosquitoes)			Contribution to the pool of infected mosquitoes before adjustment for mosquito exposure (%)			Contribution to the pool of infected mosquitoes after adjustment for mosquito exposure (%)		
Age (in years)		< 5	5 - 15	> 15	< 5	5 - 15	> 15	< 5	5 - 15	> 15	< 5	5 - 15	> 15
Burkina Faso	Dry	8.3 (4/48)	9.0 (9/100)	2.0 (1/50)	0.7 (29/4,263)	0.8 (74/8,707)	0.2 (7/4,261)	25.8	52.5	21.7	2.9	45.8	51.3
	Wet	6.7 (3/45)	7.0 (7/100)	6.0 (3/50)	2.2 (40/1,832)	1.8 (73/3,979)	0.4 (8/1,893)	32.9	45.0	22.1	3.9	41.1	55.0
Mbita	Dry	4.9 (2/41)	5.1 (3/59)	2.0 (2/102)	0.1 (2/1,468)	0.9 (21/2,292)	0.1 (5/3,311)	6.3	69.3	24.4	2.9	73.5	23.6
	Wet	0 (0/49)	2.0 (1/50)	1.0 (1/101)	0 (0/1,937)	0.2 (3/1,674)	0.06 (2/3,231)	0	57.5	42.5	0	59.7	40.3
Kilifi	Wet	3.0 (2/66)	0 (0/76)	0.8 (1/132)	0.2 (3/1,850)	0 (0/2,024)	0.03 (1/3,842)	64.1	0	35.9	46.0	0	54.0

Figure 3. Associations between gametocyte and parasite densities, gametocyte density and infectivity, and mosquito infection prevalence and infection burden. In **a**, the association between gametocyte and parasite densities is shown; the y axis is on log₁₀ scale and parasite (*I8S* qPCR) and gametocyte (*Pfs25* mRNA QT-NASBA) densities were set to 0.01 parasites (or gametocytes) per µL for negative samples to be included in this panel. In **b**, the proportion of mosquitoes infected in individual feeding experiments (y axis) and gametocytes densities (x axis) are shown. Data from all surveys are presented: gametocytes densities were quantified by *Pfs25* mRNA QT-NASBA in samples collected in Burkina Faso and Kilifi, and by microscopy for Mbita participants. Green circles correspond to samples with patent gametocytes. Both the x axis and the segment of the y axis that ranges from 0.01 to 1 are in log₁₀-scale. Individuals who did not infect mosquitoes are presented in a separate segment of y axis that only includes the 0 y-coordinate. One infectious individual from Mbita who infected 4/60 mosquitoes had no gametocytes detected by microscopy and no available sample for molecular assays and is therefore not represented in the graph. In **c**, mean and maximum (vertical line) oocyst counts per assay in experiments with at least one mosquito infection are presented. The dotted line represents the hypothetical situation if all infected mosquitoes in an experiment would have exactly one oocyst. In **b** and **c**, feeding experiments where participants had evidence of non-falciparum malaria infections were excluded.



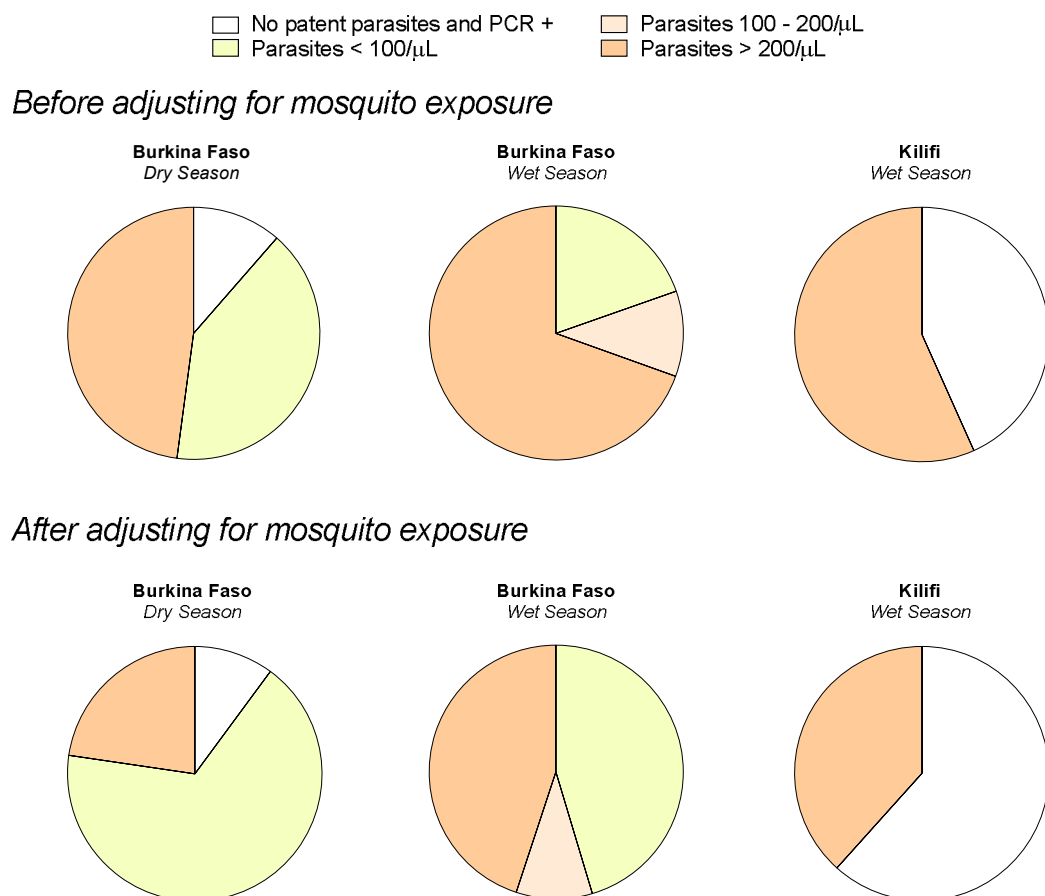
At community level, the proportion of individuals who were able to infect mosquitoes was related to transmission intensity: prevalences of infectiousness in Laye, Balonghin, Mbita dry season, Mbita wet season, and Kilifi wet season were 4.9, 6.4, 3.3, 1.1 and 0.9%, respectively, after age standardisation based on estimates for Sub-Saharan Africa [19]. In Laye and Mbita, children younger than 15 years represented the majority of infectious individuals during dry season (76.7 and 65.8%, respectively), whereas during wet season, adults and children contributed similar numbers of infectious individuals to the local infectious reservoir. Estimations that consider age-specific mosquito infection rates in feeding assays and demographic data indicate that children below 15 years of age were responsible for most (~78%) mosquito infections in the high endemicity setting in Burkina Faso (**Table 3**). The relatively high frequency of exposure to mosquitoes of adults indicates that, in Burkina Faso, adults, when infectious, have on average more opportunities to transmit infection compared to children. To estimate the proportion of all mosquito bites in a community on adults, population age structure and age-specific relative exposure to mosquitoes as estimated by our blood meal analyses were used. Age-specific probability of infection transmission in a single blood meal, determined in feeding experiments, was then used to calculate the proportion of human-to-mosquito transmission events that starts in infected adults. In Burkina Faso, this adjustment for mosquito exposure resulted in a marked change in age-specific contributions to infected mosquitoes with adults contributing ~50% of all transmission events (**Table 3**).

Infectivity to mosquitoes in relation to diagnostic performance

In our research settings, expert microscopy alone was sufficiently sensitive to detect infections in most infectious individuals (34/39 for all positive feeding assays and 28/31 when excluding non-falciparum [co-] infections). In routine practice, the lowest parasite densities detectable by routine microscopy and rapid diagnostic tests (RDTs) are estimated as 100 and 200 parasites per μL , respectively. When expressing the contribution to the infectious reservoir incorporating these theoretical limits of detection, our findings suggest that routine microscopy and RDT would detect infections in nearly half of all infectious individuals in the different settings studied (**Table S2**). The proportion of infected mosquitoes is the transmission endpoint that is of more relevance to public health. Before adjusting for mosquito exposure, 19.6 –

52.1% of *P. falciparum* infected mosquitoes became infected from individuals with *P. falciparum* parasite densities below 100 parasites per μL by microscopy, including individuals who had subpatent parasites detectable by PCR (**Figure 4**, top panels). After adjusting for mosquito exposure, these percentages increased to 45.4 – 77.3% (**Figure 4**, bottom panels).

Figure 4. Proportion of infected mosquitoes by parasite density. Age-specific prevalences of falciparum malaria parasites by microscopy and PCR and infectiousness prevalences by microscopy-defined parasite density were used to estimate the proportions of *P. falciparum* infected mosquitoes in each community; demographic age structure in Sub-Saharan Africa populations was used to standardise estimates. Individuals with evidence of non-falciparum malaria infections were excluded (N=2). The top panels represent the contributions of human infections with different parasite densities to local mosquito infections, after adjusting for population age structure and age-and-parasite density-specific probabilities of mosquito infection in feeding assays; in the bottom panels, age-specific relative mosquito exposure data were used. These calculations were based on 13, 12 and 3 infectious individuals and 108, 104 and 4 infected mosquitoes in the Burkina Faso dry and wet season surveys and in the Kilifi wet season survey, respectively. Data from Mbita are not presented as most infectious individuals in this setting had *P. malariae* co-infections.



Discussion

Quantifying the transmission potential of individuals infected with human pathogens is important to guide control strategies. Here, we report the first multi-region assessment of malaria transmission using a standardised mosquito feeding protocol and highly sensitive molecular assays for parasite and gametocyte quantification. We observed that only a small proportion of individuals living in malaria endemic areas are infectious to mosquitoes at a given time, and that this proportion decreases with decreasing transmission intensity. While children were more infectious than adults in most surveys, adults were more likely to get bitten by mosquitoes than children and consequently individuals aged > 15 years contributed considerably to falciparum infections in mosquitoes.

We performed 1,209 mosquito membrane feeding experiments in 1,075 individuals of all ages in areas with transmission ranging from intense to low (microscopy-based *P. falciparum* parasite prevalence in participants aged 2 – 10 years of 34.0, 51.0, 40.0 and 15.5% in Laye, Balonghin, Mbita during wet season, and Kilifi during wet season, respectively). Overall, we observed that between 1.0 and 7.1% of study participants were capable of infecting mosquitoes at the moment surveys were conducted. This is broadly in line with the limited data available on the human infectious reservoir [14] that are almost exclusively from areas of intense malaria transmission and used both membrane feeding and direct skin feeding assays to measure infectivity. The few studies directly comparing skin feeding versus membrane feeding, reviewed in [20], were mostly based on microscopically detectable (high-density) gametocyte carriers and suggest that skin feeding is more sensitive. Higher infection rates in skin feeding assays are nevertheless strongly correlated with infection rates measured by membrane feeding [20]. Skin feeding is biologically attractive since it best reflects natural feeding but is ethically fraught due to discomfort, particularly in children. It is currently unknown what fraction of low-density infections may result in mosquito infections in skin feeding assays but not membrane feeding assays; this information may be of great relevance to translate assessments of transmission by membrane feeding assays to the natural situation. It is noteworthy that our estimates of the proportion of infectious individuals in Burkina Faso in 2013-2014 (4.9 and 6.4%) are lower than those previously estimated in the same setting in 2007-2008[12]. In the previous study,

transmission intensity was higher than in the current study with 83 – 94 % parasite prevalence by RNA-based methods and 11 – 21% of all participants carrying microscopically-detectable gametocytes. Lower prevalence and density of parasites will have contributed to the observed difference. Temporal variation in the susceptibility of the mosquito colony to *P. falciparum* may also have contributed. One of the strengths of the current study was that we did not select individuals based on parasite status. Prior screening by molecular assays may have increased the proportion of study participants that was infectious to mosquitoes but would have left uncertainties about the transmission potential of undetected infections [6, 21]. We therefore recruited participants for feeding assays from the general population and successfully used molecular diagnostics in 3 of 4 study sites. In our surveys, all infectious individuals with molecular assays results available had parasites detected by *18S* qPCR and *Pfs25* mRNA QT-NASBA, except one infectious individual believed to have transmitted *P. malariae* parasites. This suggests that these assays might be useful to exclude non-infectious individuals. However, it is currently unclear what the kinetics of parasite densities are in chronic submicroscopic infections and conceivable that some infections that are not detectable by these sensitive assays at one time-point may increase in density and likelihood of transmission in the future.

There is accumulating evidence that in all endemicities substantial proportions of falciparum infections are subpatent, i.e. below the limit of detection of conventional field diagnostics [7]. In line with this, we detected a considerably larger number of infections with molecular assays than microscopy. In contrast to findings with high-volume qPCR from a large epidemiological study in Southeast Asia [6], where the percentage of undetectable infections was estimated based on distributions of quantifiable parasite densities, we found no evidence for a significant number of infections being missed by *18S* qPCR, as indicated by **Figure 1b**. There is considerable interest in quantifying the contribution of low density, submicroscopic, infections to onward transmission. Submicroscopic infections are defined as infections that are detectable by molecular methods but not by microscopy. In practice, this definition is influenced by the sensitivity of microscopy and molecular assays, both of which can vary between settings. In our two surveys in Burkina Faso, parasite densities below 100 parasites per μL were detected by research quality microscopy in 35.2 and 41.5% of infectious individuals, who were responsible for 45.4 and 67.2% of infected

mosquitoes (**Figure 4**), suggesting that a non-negligible proportion of transmission events may be missed by routine microscopy but not necessarily by research microscopy where a larger number of microscopic fields are screened (200 – 400 fields in our study). Both parasite quantification by microscopy and qPCR have limitations [22, 23] and ultimately the detectability of the infectious reservoir may need to be judged against diagnostic practices that are relevant to guide interventions in the field. If transmissible low-density infections could be targeted by interventions using improved diagnostics, such as highly sensitive RDTs, or that include individuals irrespective of parasite status, transmission might be reduced more effectively and rapidly. Of note, in Kilifi, one individual of three who were infectious in feeding experiments did not carry patent parasites. Whilst we believe the low proportion of infectious individuals accurately reflects the low likelihood of transmission in this setting, numbers are limited to draw conclusions on the performance of different diagnostics to identify the human infectious reservoir for malaria. For this, the methodology for xenodiagnostic studies may need to be refined to include sensitive screening tools to identify potentially infectious individuals in low transmission areas and provide more robust estimates of population infectiousness.

The probability of infecting at least one mosquito and the proportion of infected mosquitoes in successful feeding experiments were positively associated with gametocyte density [21, 24]. Among infectious individuals with *P. falciparum* mono-infection, 61.3% were gametocyte positive by microscopy despite screening 200 microscopic fields specifically for gametocytes, whilst 100.0% were gametocyte positive by *Pfs25* mRNA QT-NASBA. In our surveys, mosquito infection rates were loosely associated with gametocyte densities [21, 24] and most infectious individuals had an estimated density of 10 or more gametocytes per μL by *Pfs25* mRNA QT-NASBA. Since children harbour the highest parasite and gametocyte densities, they are generally considered to constitute a large fraction of the infectious reservoir.

Our observations also confirm that children are more likely to infect mosquitoes than adults. In the high endemicity setting in the current study, the proportion of mosquitoes acquiring malaria infection in feeding experiments was four-fold higher when feeding on blood from children below 15 years of age compared to older individuals (**Table 3**). This is in broad agreement with previous findings from the same setting (five-fold

higher infection rates in children) [12]. In the lower endemicity settings in the current study, mosquito infections were rare and occurred from children and adults without any obvious age-dependency; however, in Kilifi the number of mosquitoes dissected per assay was comparatively low, particularly during the dry season survey, which could have prevented the identification of age-related patterns of infectiousness and may have resulted in a lower sensitivity to detect sporadic mosquito infections.

One of the novel elements of our study is that we determined actual *Anopheles* mosquito exposure in areas where xenodiagnostic surveys were performed. Earlier field quantifications of human attractiveness to malaria vectors [25-27] utilised a series of different techniques, ranging from direct observation to blood meal typing using markers with limited polymorphism (e.g., ABO group). Our study expanded these observations by collecting mosquitoes in households with variable numbers of inhabitants in two different endemic settings. We were able to uniquely link 1,094 mosquito blood meals to household occupants. We observed different patterns in mosquito feeding choices in Balonghin (Burkina Faso) versus Mbita (Kenya) that have consequences for our interpretation of the human infectious reservoir for malaria. In Balonghin, adults were twenty times more likely to be sampled by mosquitoes than children aged < 5 years; in Mbita, adults received twice as many bites compared to young children. The difference in mosquito feeding choices between the sites may be partially explained by differences in bed net use; in Mbita, adults reportedly slept under nets more often (87.7%) than children < 5 years (77.8%) and schoolchildren (63.8%), while in Burkina Faso, reported bed net usage was similar in all age groups (90.0, 87.1 and 88.6% for young children, schoolchildren and adults). Differences in species composition of local vector populations is another possible explanation, although evidence for between-*Anopheles* species variation in feeding behaviour with regards to human hosts at the individual level is limited [28]. Although we also observed considerable variation in mosquito exposure between households, and ideally we would have been able to quantify mosquito exposure for every xenodiagnostic survey participant, our observations of mosquito feeding preferences in a selection of households allow a better interpretation of mosquito feeding experiments by extrapolating from standardised numbers of mosquitoes in feeding assays to actual mosquito sampling rates that are strongly age-dependent. When adjusting our estimates for mosquito exposure, the relative contribution of adults to the infectious reservoir

nearly doubles in Burkina Faso and resulted in a more modest increase in the contribution of older individuals to the infectious reservoir in our Kenya sites. Taken together, our results highlight that even in highly endemic settings where children account for the vast majority of clinical malaria episodes, control interventions that target based on infectiousness may require inclusion of adults.

Two unanticipated findings were the high proportion of *P. malariae* co-infections in Mbita and, to a lesser extent, in Burkina Faso and the high proportion of multiple-human-source blood meals in wild-caught mosquitoes. We only determined non-falciparum malaria co-infections based on microscopy. Screening for non-falciparum infections by molecular methods may have increased the estimates of the parasitaemic reservoir of *P. malariae* and *Plasmodium ovale* [29] (see *Supplementary Information*).

Overall, 14.5% of all mosquito blood meals contained DNA from more than one human source, indicative of repeated (potentially interrupted) feeding during the night prior to sampling. This relatively high percentage of bloodfed mosquitoes with multiple human sources of blood is likely to impact comprehensive malaria transmission models [30]. Currently these do not explicitly account for multiple feeding behaviour, despite previous reports [31, 32]: if sporozoites are inoculated with every probing event, mosquito multiple feeding behaviour is likely to increase the risk of human malaria infection. A number of mosquito blood meals (14.4% in Balonghin and 25.2% in Mbita) could not be linked to residents of study houses. In Balonghin, nearly all household occupants provided a blood sample that allowed genetic matching to mosquito blood meals and this suggests indoor resting of mosquitoes that fed elsewhere. In Mbita however, the higher percentage of unmatched mosquitoes could be at least partially explained by the fact that 13.3% (25/188) of household occupants did not provide blood samples for matching. A limitation of our mosquito exposure estimates is that only malaria vectors resting indoors were collected and outdoor biting may account for a non-negligible proportion of mosquito exposure events. One might assume that outdoor mosquito exposure is associated with increasing age, reflecting sleeping patterns, and our finding of a disproportionate number of mosquito bites encountered by adults may thus be an underestimate of true differences in mosquito exposure between age groups.

Individuals recruited into the xenodiagnostic surveys, as with most community-based cross-sectional surveys, were primarily asymptomatic. One study subject in Laye developed acute malaria that required immediate treatment prior to feeding assay and was not sampled, whilst three study participants in Balonghin reported recent (within a week of enrolment) antimalarial treatment, including two individuals, one infectious and one non-infectious, with *Plasmodium ovale* co-infection. In Kilifi, five individuals had positive RDT results (parasite densities by microscopy, range 1,240 – 21,500 parasites per μL) and body temperatures higher than 37.5°C; one of these individuals infected mosquitoes in feeding assays, the others participated in feeding experiments but were non-infectious. Data on recent or current malaria symptoms were not collected for Mbita participants. Our observation that gametocyte carriage is common in asymptomatic infections, 44.2 – 90.0% by *Pfs25* mRNA QT-NASBA in those individuals with *18S* qPCR positive results in our surveys, together with evidence from a previous study in Mbita where asymptomatic gametocytaemic children were substantially more infectious than symptomatic gametocytaemic children [33], suggest that individuals without malaria symptoms are likely to contribute more to local transmission compared to symptomatic individuals. Acute symptomatic infections with high parasite densities are likely to be characterised by a shorter duration of infection and thus shorter time-window that allows gametocyte production compared to infections that are more chronic in nature[34]. Despite these considerations that support the importance of asymptomatic infections for the human infectious reservoir, it is of great relevance to directly compare onward transmission from symptomatic and asymptomatic infections in the same setting. Such studies require a design where participants are recruited from both local health facilities and the communities they serve.

In summary, our study that combined actual mosquito exposure assessments and xenodiagnostic surveys indicates that approximately 45 – 75% of all mosquito infections are caused by individuals with total parasite densities below 100 parasites per μL but gametocyte densities above 10 gametocytes per μL . These densities are not generally detected by routine diagnostics. Because of their higher exposure to mosquitoes, adults contribute much more to local transmission in some settings than expected based on age-specific infectiousness prevalences and adjustments for population age structure. These findings highlight the potential roles for sensitive

diagnostics and interventions that target all age groups to reduce the transmission of malaria across different endemicities.

Methods

Infectivity studies - study areas and populations

Our surveys were conducted in an area of intense malaria transmission in Burkina Faso and areas of low and moderate malaria transmission in Kenya. In Burkina Faso, the dry season survey was performed in the village of Laye and the wet season survey in nearby Balonghin. Both villages are characterized by intense seasonal transmission [12, 35]. In Kenya, dry and wet season surveys were performed in Mbita, on the shores of Lake Victoria in the Suba District in Western Kenya, an area characterised by moderate malaria transmission intensity [36]. In Kilifi, in coastal Kenya, malaria transmission occurs throughout the year, but peaks during the wet season [37]. Over the last 25 years, malaria incidence in this area has declined to very low levels [38]. Of note, health facilities, clinics, were available in all study villages, and artemisinin-based combination therapy is used as first-line treatment of clinical cases in all sites. At the time of these surveys, seasonal malaria chemoprophylaxis for children under 5 years of age was not part of national guidelines in Burkina Faso.

In all xenodiagnostic surveys, children aged 2 years or older and adults were recruited regardless of their parasite status. Individuals with serious clinical conditions requiring immediate treatment were not eligible and were referred to the nearest health facility. Different population sampling strategies were adopted in different surveys. In Laye, recent census data were not available, and the random walk method [39] was used to select households (N=59); each selected household contributed 4 (median, IQR 3 – 4) study participants, children and adults. In Balonghin (Burkina Faso), a census list was used to randomly select households (N=81); individuals living in these households were randomly selected to participate in the study. For the cross-sectional study performed in Mbita during the dry season, a different sampling strategy was used: the study area was divided in sub-areas with ~10 households; the study team visited a sub-area per day and randomly selected up to six subjects to membrane feeding experiments among those individuals willing to participate in the study. During the

following wet season, whenever possible, the same individuals that participated in the first Mbita survey were recruited. In Kilifi, individuals were invited to come to the study clinic and, as with the other study sites, were enrolled regardless of their parasite status.

Infectivity studies - sampling strategy

The objective of these surveys was to estimate the prevalence of infectiousness in different age groups and transmission settings. Due to logistical constraints related to mosquito husbandry and dissection capacity, we estimated that we would be able to perform on average 6 membrane feeding assays per day in the different study sites. Two hundred participants were recruited during each survey in Burkina Faso and Mbita; in Kilifi, seasonality is less marked and 413 individuals were recruited over a period of 14 months. Children aged 5 years or younger, schoolchildren (between 5 and 15 years) and adults (15 years or older) were recruited in a 1 : 2 : 1 ratio in surveys performed in Burkina Faso; in Kilifi and Mbita, where adults were hypothesized to form a more important part of the parasitaemic reservoir, a ratio of 1 : 1 : 2 was targeted.

Infectivity studies - Parasite detection

Light Microscopy

Malaria parasites (asexual and sexual stages) were quantified by microscopy. All slides were double-read and considered negative if no parasites were detected in 100 (Burkina Faso and Kilifi) or 200 (Mbita) microscopic fields. Densities of falciparum and non-falciparum malaria parasites were determined by assuming 8,000 white blood cells per μL of blood.

Molecular assays

Finger prick blood samples collected in Laye, Balonghin and Mbita were stored in RNAprotect Cell Reagent® and had nucleic acids extracted using MagNAPure LC automatic extractor (Total Nucleic Acid Isolation Kit – High Performance, Roche Applied Science). All samples from Kilifi were collected by venipuncture: DNA was extracted from 100 μL of whole blood using the Qiagen extraction method on an

automated Qiaxtractor (Qiagen); for RNA based assays, 100 μ L of whole blood was stored in the appropriate volume of Trizol (Invitrogen) and extracted using the phenol-chloroform method. *18S* rRNA quantitative nucleic acid sequence-based amplification (*18S* QT-NASBA) was used to detect all falciparum parasite stages. Nested PCR targeting the *18S* rRNA gene was also performed to detect falciparum infections [40]; and quantitative PCR (*18S* qPCR) allowed estimation of *P. falciparum* parasite burden. The detection and quantification of mature *P. falciparum* gametocytes was performed by *Pfs25* mRNA QT-NASBA according to the protocol described in [24].

Infectivity studies - assessment of infectiousness

Venous blood samples collected in heparinized tubes were used for whole blood membrane feeding assays following an established protocol [41]. Immediately after venipuncture, 400-500 μ L of blood were offered to female *A. gambiae* mosquitoes via an artificial membrane. After 20 minutes, fully fed mosquitoes were transferred to storage cups by aspiration and kept at 29°C on average for one week (6 – 8 days) with access to glucose solution prior to dissection and microscopical assessment for the presence of oocysts.

Mosquito infections in Burkina Faso and Kilifi surveys were confirmed by molecular methods. Midguts of mosquitoes with at least one oocyst identified by microscopy were stored in 50 μ L of RNAlater Cell Reagent®. *MSP2*-based nested PCR [42] was used for mosquitoes infected in Burkina Faso feeding experiments. Genetic material (cDNA) of mosquitoes infected in Kilifi were tested with *CSP*-based PCR. We were not able to reliably confirm mosquito infection for Mbita feeding experiments due to technical issues with sample storage.

Mosquito exposure quantification - indoor mosquito collections

Mosquito collections were performed in Balonghin (Burkina Faso) and Mbita (Kenya). In Balonghin, mosquito collections were performed at three different timepoints: end of the 2013 wet season (November – December), and start (June 2014) and peak (September 2014) of the following wet season. Every week indoor mosquito collections were performed in five households. During the first entomological survey (2013), 40 randomly selected households were included; for each household,

mosquitoes were collected during seven days or until 30 blood fed mosquitoes were collected. In the second and third surveys, mosquitoes were collected over 5 days in 20 households with highest mosquito exposure in the first survey and with no changes in the number of inhabitants. Demographic data are not available for 5 houses selected only for the 2013 survey and where no bloodfed mosquitoes were obtained; individuals living in these households are not included in the analysis presented here. In Mbita, mosquitoes were collected from 40 households during a single time-point from August to December 2015; mosquitoes were collected during seven days or until 30 blood fed mosquitoes were collected.

Mosquitoes were aspirated from walls and ceilings between 7 and 9 in the morning for a maximum of 15 minutes per house, transferred to paper cups, transported to the Centre National de Recherche et de Formation sur le Paludisme (CNRFP) in Ouagadougou or the International Centre of Insect Physiology and Ecology (ICIPE) in Mbita and processed immediately upon arrival to minimise DNA degradation. If blood fed, the abdomen of mosquitoes was squeezed onto a Whatman 3MM filter paper for later DNA extraction. To link mosquito blood meals to specific individuals, all permanent inhabitants of households selected for mosquito sampling were asked to provide a single finger prick blood sample into plastic K2EDTA BD Microtainer® for collection of 50 µL-blood samples in 250 µL of RNAprotect Cell Reagent® and onto a Whatman 3MM filter paper.

Mosquito exposure quantification - identification of blood meal source

Nucleic acids were extracted from filter papers with squeezed bloodfed mosquito abdomens and with human samples from Balonghin study participants using Boom extraction method [43]. Nucleic acids from human blood samples collected in Mbita in RNAprotect Cell Reagent® were extracted using MagNAPure LC automatic extractor (Total Nucleic Acid Isolation Kit – High Performance, Roche Applied Science).

Molecular analysis of blood meals was performed using Authentifiler™ PCR Amplification kit (Applied Biosystems®), which consists of a multiplex PCR assay with 9 microsatellite markers and one gender-determining marker (Amelogenin). Automated capillary electrophoresis was used to determine DNA profiles in mosquito blood meals. 88/1,066 and 72/808 mosquito samples collected in Balonghin and Mbita

respectively had no amplification. A small number of samples with amplification had less than 10 alleles detected (12 in Balonghin and 47 in Mbita) and were not included in our analysis. 153 mosquito samples from Balonghin and 87 from Mbita had evidence of multiple human DNA sources, i.e. more than two alleles in at least three loci (see *Results* section and *Supplementary Information*, including **Tables S3 and S4**). This conservative definition of multiple feeding was to avoid artefacts due to minor peaks in capillary electrophoresis resulting from variable amounts of human DNA. Of those mosquito blood meal samples that had a single human DNA source, 139 and 174 in Balonghin and Mbita respectively were not matched to someone living in the same household where they were collected and for this reason were also excluded from our analysis; additionally 8 mosquitoes collected in Burkina Faso did not have identification data available.

Statistical Analysis

Statistical analyses were performed in Stata 14® (StataCorp LP, Texas, USA). Prevalences of falciparum parasites and gametocytes were estimated for each xenodiagnostic survey. Age-specific (< 5 years, 5 – 15 years and > 15 years) asexual stage parasite, gametocyte and infectivity prevalences were also calculated. We compared parasite levels between children and adults and gametocyte levels during wet season surveys versus dry season surveys using negative binomial regression. Logistic regression was used to assess the association between age and infectiousness (at least one infected mosquito in feeding experiments). In these models, estimates were adjusted for study site. In **Figure 4**, parasites densities in patent infections were defined based on microscopy results; the highest stage-specific density (asexual stage levels or gametocytaemia) in individual infections was used. In **Figure 1**, two participants of the Kilifi wet season survey had parasite densities above 100,000 parasites per μL by *18S* qPCR, ~10 – 30 times higher than microscopy-based estimates, and microscopy-defined densities were presented for these individuals. In **Figure 2**, fractional polynomials were used to determine the combinations of powers that best describe the associations between age or body surface area and number of mosquito bites; mixed effects negative binomial models with household as random effect were fitted. The lines in **Figure 2** panels **b-d** correspond to the predicted number

of mosquito bites per individual; for Balonghin (panels **b** and **d**), this number represents mosquito exposure during peak wet season over five days.

An algorithm was developed that quantified squared differences in allele sizes for each locus between human and mosquito samples and identified study participants with the lowest mean allelic distance to each mosquito blood meal. All blood meal samples from the Burkina Faso study and a subset of mosquito samples from Mbita had their matching result checked manually. The effect of age, as a categorical variable, on mosquito exposure (i.e., the number of singly matched mosquito blood meals to an individual) was assessed by mixed effects negative binomial regression. Initially, we fit a model that included data from both study sites and tested for interaction between age effects and study site effects. Since there was a significant interaction ($P = 0.001$) between study site and age effects, we present separate analyses for the different sites. For the entomological study in Balonghin, this analysis was adjusted for survey (fixed effect). To account for data correlatedness and household-level differences in vector abundance, the models for both sites had household as random effect.

Age-specific contributions to transmission were estimated as (i) the proportion of infectious individuals from each age group and (ii) the proportion of infected mosquitoes acquiring infection from each age group.

The relative contribution (C_i) of each age group i to the pool of infective individuals in each study site was calculated:

$$C_i = p_i d_i / \sum_1^n p_i d_i \quad [1]$$

where d_i is the proportion of the population in age group i , p_i is the age-specific prevalence of infectivity (at least one infected mosquito in feeding experiments), and n is the number of categories into which the population was divided. Values of p_i are study site-specific. For this analysis three age categories were used ($n = 3$, < 5 years, $5 - 15$ years and > 15 years) and we used an age structure based on recent estimates for Sub-Saharan Africa [19].

To estimate the proportion of infected mosquitoes that acquire their parasites from a specific age group (M_i) (**Table 3**), the following formulas were used:

$$b_i = d_i a_i / \sum_1^n d_i a_i \quad [2]$$

$$M_i = w_i b_i / \sum_1^n w_i b_i \quad [3]$$

where b_i is the proportion of all mosquito bites which occur on age group i , and a_i represents the age-specific relative exposure to mosquitoes, which was calculated based on the results of mosquito blood meal analysis (for Burkina Faso study sites, $a_1 = 1$, $a_2 = 7.7$, $a_3 = 20.9$; for Mbita surveys, $a_1 = 1$, $a_2 = 2.3$, $a_3 = 2.1$); w_i corresponds to the probability of mosquito infection (number of mosquitoes infected divided by total number of mosquitoes dissected) in feeding assays involving individuals from age group i , regardless of their parasite status.

We also determined the contribution to transmission of individuals with different parasite densities (M_j) (**Figure 4**). Equation 3 was modified by re-assigning mosquito bites by age and diagnostic status using age-specific prevalences of infections with different parasite levels (t_{ij}), where i corresponds to specific age groups and j to parasite density categories:

$$M_j = \sum_{i=1}^n w_{ij} b_i t_{ij} / \sum_{j=1}^m \sum_{i=1}^n w_{ij} b_i t_{ij} \quad [4]$$

Here, m represents the number of different diagnostics categories: $j = 1$ (no patent parasites but PCR positive), $j = 2$ (patent infections with < 100 parasites per μL), $j = 3$ (patent infections with $100 - 200$ parasites per μL) or $j=4$ (patent infections with >200 parasites per μL). In this modified framework, w_{ij} represents the age (i) and parasite density category (j) specific probability of mosquito infection in feeding assays.

Ethics

The study received ethical clearance from local and international ethical committees: xenodiagnostic and entomological studies in Burkina Faso were approved by the ethical review committees of the Ministry of Health of Burkina Faso and of the London School of Hygiene and Tropical Medicine (reference numbers 6271 and 6447); xenodiagnostic surveys in Mbita and Kilifi, Kenya, were combined in one protocol and received clearance from the Kenya Medical Research Institute Scientific Ethics Research Unit (KEMRI-SERU SSC number 2574). Written informed consent was obtained from all participants or the parents/guardians.

Data availability

The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

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Acknowledgments

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Contributions

C.D. and T.B. designed the study. B.G., M.K., C.D. and T.B. wrote the first draft of the manuscript. B.G., M.K., J.B. and T.B. analysed the data. B.G., W.M., A.T., I.N., S.S. and T.B. contribute to data collection in Burkina Faso. M.K., J.N., D.M., J.M., K.M and P.B. contributed to data collection in Kilifi. P.S., W.S., G.B., R.O. and M.N. contributed to data collection in Mbita. K.L., J.N. and D.M. performed molecular assays for parasite detection and quantification. L.G. performed mosquito blood meal genotyping; J.H. developed algorithm used to match blood meals to humans. C.D. and T.B. led the study team. All authors contributed to interpretation of the analyses and revised the draft manuscript.

Supplementary Information

Quantification of parasites

We observed a strong association between *18S* qPCR parasite densities and microscopy parasite densities. Where both assays detected parasites, microscopy overestimated parasite densities compared to *18S* qPCR (**Figure S1**). One possible explanation for this overestimation is the assumption made during microscopy-based quantification that white blood cells levels are equal to 8,000 cell per μL . In one of the study sites in Burkina Faso (Balonghin), 34.4% of children recruited to a recent primaquine trial had white blood cell counts per μL of blood below 6,000 at enrolment (Gonçalves, personal communication).

Non-falciparum malarias and their potential infectiousness

Microscopically-detectable non-falciparum malaria parasites were present in all study sites. Patent *Plasmodium malariae* infections were more common in Mbita (5.9 [12/202] and 7.9 [16/202] % prevalence during dry and rainy seasons, respectively); only one individual in Kilifi had *P. malariae* co-infection. In Laye, three participants had patent malariae parasites, whilst in Balonghin one malariae infection was observed. *Plasmodium ovale* infections (N=4) were also detected during surveys in Balonghin and Mbita (wet season). In total, 29 individuals had more than one malaria species identified in blood smear.

Overall, 8 of the 39 infectious individuals had patent non-falciparum malaria parasites (**Table S1**). In Laye, one individual who infected 2/77 mosquitoes had patent malariae gametocytes and no evidence of falciparum infection by microscopy or molecular assays. In Balonghin, one individual who infected 17/35 mosquitoes had both patent ovale and falciparum gametocytes. In Mbita, 5/9 infectious individuals carried both patent falciparum and malariae parasites and one, only malariae parasites, including gametocytes.

Sensitivity analysis on age-specific exposure to mosquitoes in Burkina Faso

To assess whether the non-negligible number of mosquitoes with multiple human source blood meal could have influenced our results, we fit mixed effects negative binomial models similar to the model described in the *Methods* section under different assumptions. In one model, we evenly allocated mosquito bites with multiple human DNA sources to all individuals living in the households where they were collected. Under this conservative assumption, adults (incidence rate ratio [IRR] 7.7 95% confidence interval [CI] 3.8 – 15.7) and children aged 5 to 15 years (IRR 3.7 95% CI 1.8 – 7.4) were more often bitten by *Anopheles* mosquitoes than younger children (**Table S3**). In a different analysis, we assumed that individuals who had the lowest genetic distances to the genotypes present in these meals were blood sources. As most (98%) singly matched meals involved mean squared allelic distances lower than 1, this cut-off was used. Using this approach, we linked 125 multiple-source blood meals to one or two individuals in the same household, and we observed that adults (IRR 10.9 95% CI 4.6 – 25.8) and schoolchildren (IRR 4.6 95% CI 2.0 – 10.5) were more often exposed to anopheline vectors than children aged < 5 years.

Figure S1. Parasite quantification by *18S* rRNA gene qPCR and microscopy. To facilitate visualisation, y-axes (parasite densities quantified by *18S* qPCR) are in log₁₀ scale. Samples from four individuals with patent asexual stage parasites (one sample from Burkina Faso dry season survey, and three from Kilifi wet season survey) did not have parasites detected by *18S* qPCR and are not presented. The inset plots present parasitaemias when parasites were detected by both methods.

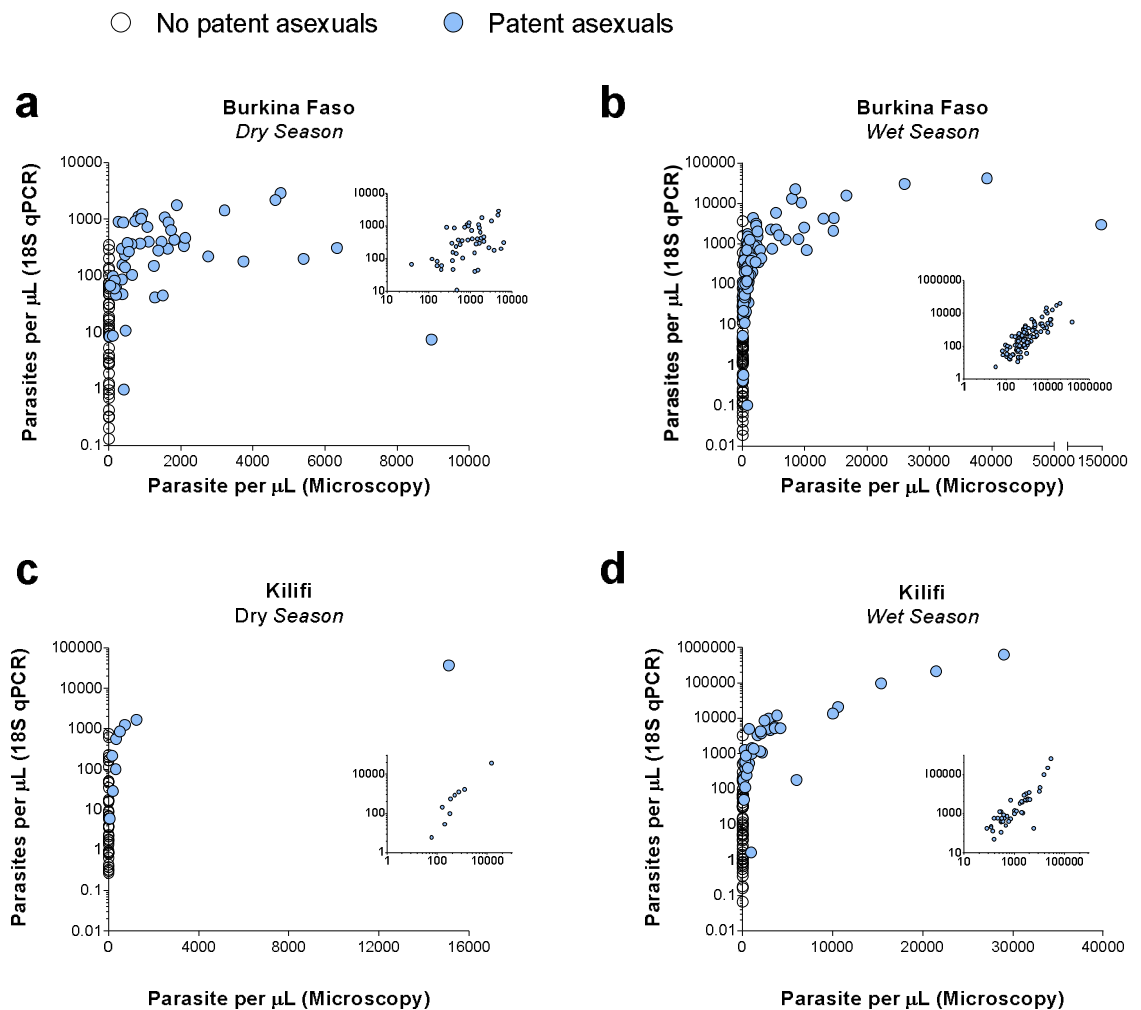


Figure S2. Age-specific prevalences of infectiousness by site and season.

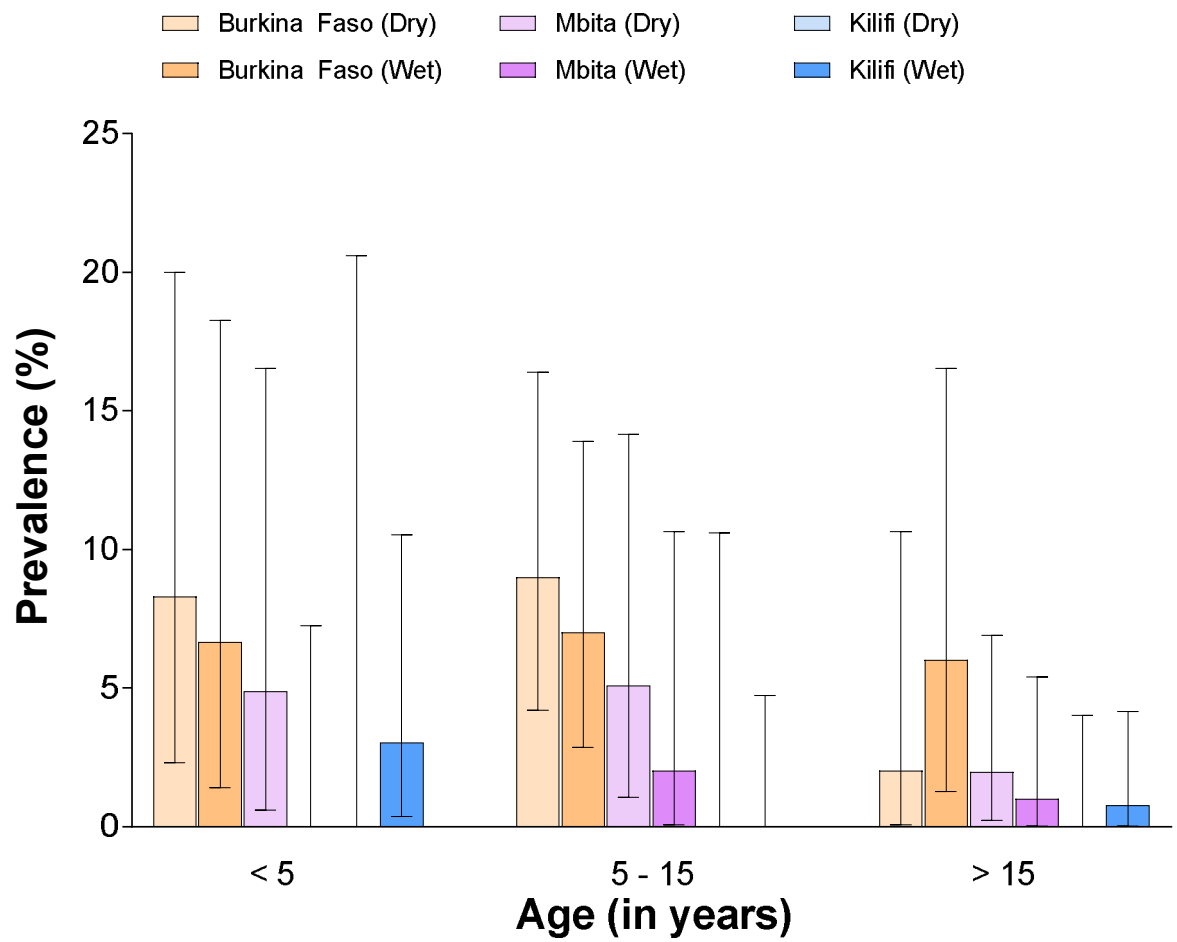


Figure S3. Infectiousness to mosquitoes and infection detectability. The percentages (y-axes) of infectious individuals with parasites detected by different diagnostic methods (x-axes) are presented.

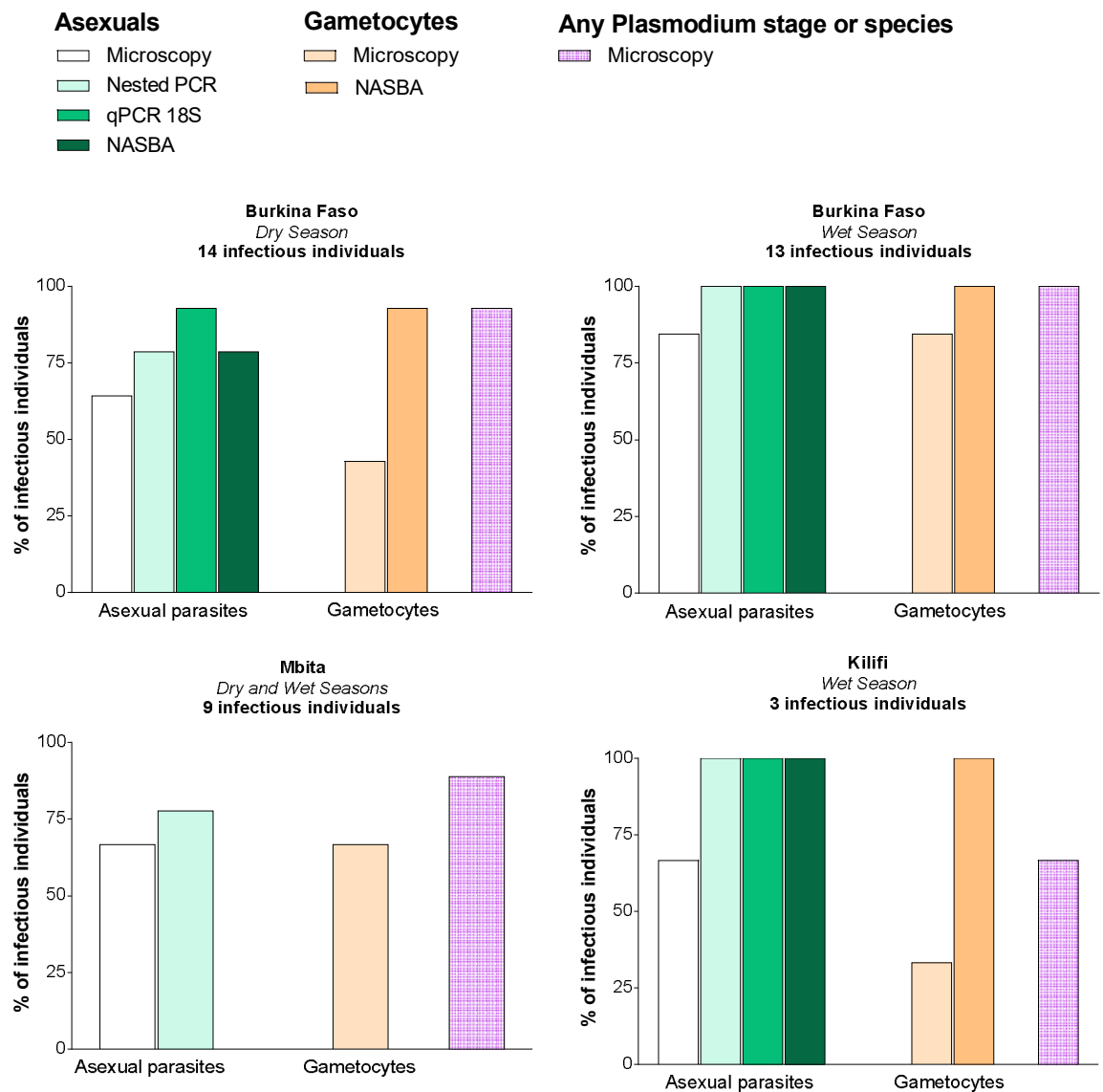


Table S1. Infectious individuals sorted study site survey, and 18S qPCR-based parasite density.

Study Site	Survey	Proportion (n/N) infected mosquitoes	Median (range) oocyst count	Patent falciparum asexual	Patent falciparum gametocytes	<i>P. malariae</i> parasites	<i>P. ovale</i> parasites	<i>Pfs25</i> mRNA QT- NASBA	<i>18S</i> rRNA QT- NASBA	<i>18S</i> qPCR	<i>18S</i> qPCR (parasites per μL)	<i>Pfs25 QT-NASBA</i> (gametocytes per μL)	Molecular confirmation of falciparum mosquito infection
Laye	Dry	0.03 (2/77)	(1, 1)	Negative	Negative	Positive	Negative	Negative	Negative	Negative	0.0	0.0	Negative
Laye	Dry	0.10 (7/71)	3 (1 – 4)	Negative	Positive	Negative	Negative	Positive	Positive	Positive	0.4	110.1	All <i>P. falciparum</i>
Laye	Dry	0.27 (21/78)	2 (1 – 5)	Negative	Positive	Negative	Negative	Positive	Positive	Positive	0.7	9.2	All <i>P. falciparum</i>
Laye	Dry	0.04 (3/73)	1 (1 – 1)	Positive	Positive	Negative	Negative	Positive	Positive	Positive	1.0	7.2	All <i>P. falciparum</i>
Laye	Dry	0.05 (3/65)	1 (1 – 1)	Negative	Positive	Negative	Negative	Positive	Negative	Positive	8.6	10.8	All <i>P. falciparum</i>
Laye	Dry	0.16 (16/98)	1 (1 – 5)	Negative	Negative	Negative	Negative	Positive	Positive	Positive	11.9	10.9	All <i>P. falciparum</i>
Laye	Dry	0.02 (2/96)	(1, 3)	Positive	Negative	Negative	Negative	Positive	Positive	Positive	150.3	50.3	All <i>P. falciparum</i>
Laye	Dry	0.20 (14/71)	3.5 (1 – 11)	Positive	Negative	Negative	Negative	Positive	Positive	Positive	200.9	88.1	All <i>P. falciparum</i>
Laye	Dry	0.28 (24/87)	2.5 (1 – 22)	Positive	Positive	Negative	Negative	Positive	Positive	Positive	218.8	42.5	All <i>P. falciparum</i>
Laye	Dry	0.03 (3/88)	2 (1 – 2)	Positive	Positive	Negative	Negative	Positive	Positive	Positive	281.2	11.0	All <i>P. falciparum</i>
Laye	Dry	0.01 (1/97)	1	Positive	Negative	Negative	Negative	Positive	Positive	Positive	399.0	24.1	All <i>P. falciparum</i>
Laye	Dry	0.02 (2/81)	(1, 1)	Positive	Negative	Negative	Negative	Positive	Positive	Positive	431.5	13.0	All <i>P. falciparum</i>
Laye	Dry	0.01 (1/76)	1	Positive	Negative	Negative	Negative	Positive	Positive	Positive	1119.4	21.4	All <i>P. falciparum</i>
Laye	Dry	0.13 (11/84)	2 (1 – 4)	Positive	Negative	Negative	Negative	Positive	Negative	Positive	1782.4	54.3	All <i>P. falciparum</i>
Balonghin	Wet	0.03 (1/39)	1	Negative	Positive	Negative	Negative	Positive	Positive	Positive	0.4	21.2	All <i>P. falciparum</i>
Balonghin	Wet	0.05 (2/39)	(1, 2)	Negative	Positive	Negative	Negative	Positive	Positive	Positive	1.3	116.9	All <i>P. falciparum</i>
Balonghin	Wet	0.33 (14/43)	2 (1 – 5)	Positive	Positive	Negative	Negative	Positive	Positive	Positive	15.1	106.2	All <i>P. falciparum</i>
Balonghin	Wet	0.05 (2/44)	(2, 2)	Positive	Negative	Negative	Negative	Positive	Positive	Positive	54.1	68.1	All <i>P. falciparum</i>
Balonghin	Wet	0.14 (5/35)	2 (1 – 5)	Positive	Positive	Negative	Negative	Positive	Positive	Positive	114.3	89.9	All <i>P. falciparum</i>
Balonghin	Wet	0.03 (1/33)	1	Positive	Positive	Negative	Negative	Positive	Positive	Positive	356.5	32.7	All <i>P. falciparum</i>
Balonghin	Wet	0.34 (17/50)	3 (1 – 16)	Positive	Negative	Negative	Negative	Positive	Positive	Positive	435.5	161.1	All <i>P. falciparum</i>
Balonghin	Wet	0.29 (15/51)	1 (1 – 15)	Positive	Positive	Negative	Negative	Positive	Positive	Positive	450.8	144.5	All <i>P. falciparum</i>
Balonghin	Wet	0.49 (17/35)	4 (1 – 15)	Positive	Positive	Negative	Positive	Positive	Positive	Positive	712.9	0.04	Negative
Balonghin	Wet	0.23 (8/35)	4 (2 – 13)	Positive	Positive	Negative	Negative	Positive	Positive	Positive	1324.3	162.5	All <i>P. falciparum</i>
Balonghin	Wet	0.27 (12/45)	4 (1 – 9)	Positive	Positive	Negative	Negative	Positive	Positive	Positive	2264.6	220.8	All <i>P. falciparum</i>
Balonghin	Wet	0.50 (24/48)	16 (1 – 71)	Positive	Positive	Negative	Negative	Positive	Positive	Positive	4305.3	3228.5	All <i>P. falciparum</i>
Balonghin	Wet	0.07 (3/41)	8 (3 – 8)	Positive	Positive	Negative	Negative	Positive	Positive	Positive	30478.9	144.9	All <i>P. falciparum</i>
Mbita	Dry	0.07 (4/60)	1 (1 – 1)	Negative	Negative	Negative	Negative	-	-	-	-	-	1 mosq. <i>P. falciparum</i>
Mbita	Dry	0.02 (1/56)	1	Positive	Negative	Positive	Negative	-	-	-	-	-	-
Mbita	Dry	0.57 (17/30)	8 (2 – 197)	Negative	Positive	Positive	Negative	-	-	-	-	-	1 mosq. <i>P. malariae</i>
Mbita	Dry	0.02 (1/44)	3	Positive	Positive	Positive	Negative	-	-	-	-	-	-
Mbita	Dry	0.02 (1/60)	1	Positive	Positive	Negative	Negative	-	-	-	-	-	-
Mbita	Dry	0.02 (1/60)	1	Positive	Positive	Negative	Negative	-	-	-	-	-	-
Mbita	Dry	0.05 (3/60)	1 (1 – 1)	Positive	Positive	Positive	Negative	-	-	-	-	-	1 mosq. <i>P. malariae</i>
Mbita	Wet	0.07 (2/30)	(1, 2)	Negative	Negative	Positive	Negative	-	-	-	-	-	-
Mbita	Wet	0.08 (3/37)	2 (1 – 4)	Positive	Positive	Positive	Negative	-	-	-	-	-	-
Kilifi	Wet	0.07 (1/15)	1	Negative	Negative	Negative	Negative	Positive	Positive	Positive	30.7	2.0	Positive
Kilifi	Wet	0.04 (2/45)	(1, 4)	Positive	Positive	Negative	Negative	Positive	Positive	Positive	1361.4	266.1	Positive
Kilifi	Wet	0.03 (1/32)	3	Positive	Negative	Negative	Negative	Positive	Positive	Positive	5345.8	107.9	Positive

Table S2. Malaria diagnostics and contribution to the local population of infectious individuals.

	Contribution to the pool of infectious individuals (%)		
	<i>Laye</i>	<i>Balonghin</i>	<i>Kilifi (Wet Season)</i>
Diagnostics			
<i>No patent parasites and PCR</i>			
+	5.7	0	46.5
< 100 parasites per μL	35.2	41.5	0.0
100 - 200 parasites per μL	0.0	4.3	0.0
> 200 parasites per μL	59.0	54.1	53.5

Table S3. Sensitivity analyses of age-specific mosquito exposure in Burkina Faso.

	Incidence Rate Ratio (95% confidence interval)		
	<i>Excluding mixed blood meal mosquitoes*</i>	<i>Including mixed blood meal mosquitoes and assuming equal exposure**</i>	<i>Including mixed blood meal mosquitoes and matching to lowest genetic distance</i>
Age (in years)			
< 5	Reference	Reference	Reference
5 – 15	7.7 (2.9 - 20.8)	3.7 (1.8 - 7.4)	4.6 (2.0 - 10.5)
> 15	20.9 (7.7 - 57.4)	7.7 (3.8 - 15.7)	10.9 (4.6 - 25.8)

*as reported in the main text; based on 666 uniquely matched mosquitoes

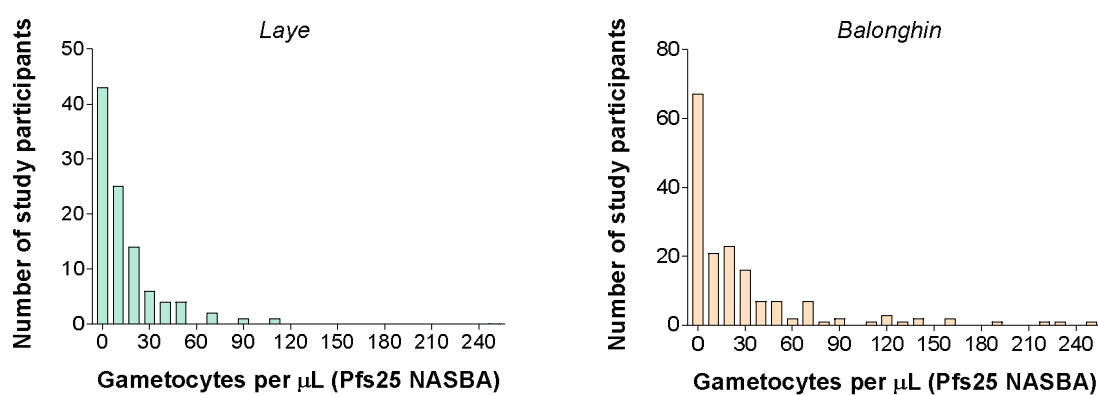
**assuming equal exposure among age groups of mosquitoes with multiple blood meals. This analysis was based on 666 singly matched mosquitoes and 153 mosquitoes with multiple human DNA sources that were allocated evenly to all individuals living in the households where they were collected.

Table S4. Distribution of multiple source blood meals by number of loci with multiple alleles (three or more alleles per loci).

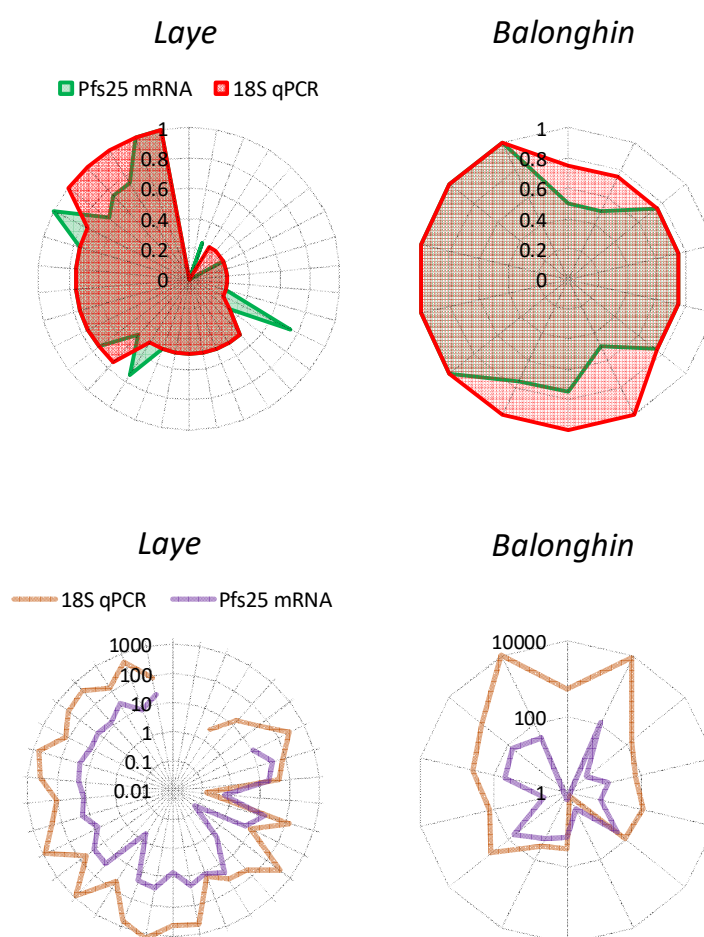
Number of loci with multiple alleles (3 or more)	Study sites	
	<i>Balonghin</i>	<i>Mbita</i>
3	45	27
4	29	13
5	38	21
6	28	8
7	11	12
8	2	2
9	0	4
10	0	0

Additional Figures

Additional Figure 1. Distribution of gametocyte densities during dry (Laye) and wet (Balonghin) seasons. Only *Pfs25* mRNA QT-NASBA results of individuals who had parasites detected by *18S* qPCR are included in this graph. 57 and 48.2% of parasite-positive individuals in Laye and Balonghin, respectively, had 10 or fewer gametocytes per μL . A total of 14 individuals had sexual stage parasites detected by QT-NASBA and negative *18S* qPCR results and are not included in this figure.



Additional Figure 2. Distribution of parasites by households during the two xenodiagnostic surveys in Burkina Faso. Only households with four study participants were included in this graph (34 houses in Laye, 14 in Balonghin). The top panels represent household-specific prevalences (radial axes) of parasites (*18S* qPCR) and gametocytes (*Pfs25* mRNA QT-NASBA). The bottom panels represent the mean densities (per μL) of parasites and gametocytes in different households; only individuals with parasites detected by molecular assays were included in the calculations of the means. The radial axes of the lower panels are in logarithmic scale (base 10). Of note, 2/27 infectious individuals, recruited in Balonghin, lived in the same house.



Chapter 4, Variation in natural exposure to anopheles mosquitoes and its effects on malaria transmission

The number of mosquito bites a malaria-infected individual receives determines his or her contribution to onward malaria transmission. In this chapter, I present data on individual-level exposure to *Anopheles* mosquitoes in a village with endemic malaria transmission in Burkina Faso. This work was submitted for publication in October 2017; an early draft is included in this chapter. An additional figure is also presented at the end of the chapter.

A revised version (December 2017) of this manuscript, with modifications suggested by reviewers, as well as our answers to reviewers' comments are included in the *Appendix*.



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SECTION A – Student Details

Student	Bronner Pamplona Augusto Gonçalves
Principal Supervisor	Chris Drakeley
Thesis Title	The human infectious reservoir of falciparum malaria

If the Research Paper has previously been published please complete Section B, if not please move to Section C

SECTION B – Paper already published

Where was the work published?	
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Stage of publication	Pending comments from other authors

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For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper	I wrote the first draft of this manuscript, with TB and CD. I performed the analyses included in this version.
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Student Signature: Bronner P. A. G. Date: 04/09/2017

Supervisor Signature: Chris Drakeley Date: 04/09/2017

Title page

Title: Variation in natural exposure to anopheles mosquitoes and its effects on malaria transmission

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Abstract

Variation in biting frequency by *Anopheles* mosquitoes can explain some of the heterogeneity in malaria transmission in endemic areas. In this study, we assessed natural exposure to mosquitoes by genotyping bloodfed resting mosquitoes and matching their blood meals with blood from residents of local households. We observed that the distribution of mosquito bites exceeded the Pareto rule (20/80) in two of the three surveys performed: 85.1, 76.0, and 95.5% of mosquito blood meals were matched to one fifth of the study population at the start, peak and end of the transmission season, respectively. Both between- and within-household differences in exposure contributed to the high number of individuals with no matched blood meals. The distribution of potential parasite inoculations (i.e., bites from mosquitoes with sporozoites detected by PCR) followed a similar pattern, with a small number of individuals receiving multiple potentially infectious bites over the period of a few days. Together, our findings indicate that some individuals are much more frequently sampled and infected by *Anopheles* mosquitoes than the rest of the population, which might explain heterogeneity in infection risk and suggests significant variation in malaria transmission potential.

Introduction

Malaria epidemiology is doubly dependent on the frequency of contacts between human hosts and *Anopheles* mosquitoes, which links the number of mosquito infections caused by an infectious human host and the rate at which uninfected humans acquire infections. Describing the variability in the frequency of human sampling by malaria vectors is therefore essential to understand parasite transmission from and to humans. While at a local level vector density determines average mosquito exposure, even within the same locality individuals may not be equally likely to be bitten by *Anopheles* mosquitoes [1-4]. Exposure to malaria vectors is influenced by host availability (i.e., amount of time an individual remains unprotected against mosquito bites in an environment where anopheline mosquitoes are present) and attractiveness to mosquitoes [5]. Availability determines when and where individuals might be sampled by mosquitoes: a multicentre study in Africa that collected entomological and human behavioural data estimated that more than three quarters of human exposure to anopheline mosquito bites occur when individuals are indoors [6]. For individuals who are accessible to malaria vectors, age and body surface area [3, 7] are two major determinants of attractiveness to mosquitoes, although other factors also play a role [2].

The multifactorial nature of mosquito exposure in malaria endemic areas indicates that, while experimental and quasi-experimental studies are valuable, they will not accurately capture inter-individual variation in actual exposure. Here, we describe the variability in real-life exposure to malaria vectors by linking, through DNA fingerprinting, blood meals of wild-caught mosquitoes to humans living in the households where they were collected. Previously we have shown that these mosquitoes fed more often on adults (Gonçalves et al, *submitted*). We now extend this analysis to assess whether mosquito bites are distributed evenly in the population or are concentrated in a few individuals and to determine the frequency of *Anopheles* species-specific mosquito bites, and potential parasite inoculations (i.e., sporozoite-positive mosquito bites) per individual.

Methods

Study area and mosquito collections

This study was conducted in Balonghin (health district of Saponé, Burkina Faso), an area characterized by seasonal malaria transmission between August and December following rainfall between June and September [8]. Enrolment coincided with a malaria treatment trial that recruited asymptomatic parasite carriers [9], and all households where children participating in that trial lived were eligible for this study.

Indoor mosquito collections were performed at the end of the 2013 transmission season (October – December), and at the start (July 2014) and peak (September 2014) of the following wet season. Every week mosquito collections were performed in five households. Forty households were included in the first survey and, for each household, mosquitoes were collected by mouth aspiration for seven days or until 30 bloodfed *Anopheles* mosquitoes were collected. In 2014, mosquito collections were performed in 20 of these 40 initial households over 10 days (5 days in July, and 5 in September). Bloodfed mosquitoes had their head-thoraces stored for speciation and malaria infection assessment by PCR (see below), and their abdomens containing blood meal material squeezed on filter paper.

Mosquito blood meal typing

Genetic typing of these blood meal samples has been described in detail elsewhere (Gonçalves et al, *submitted*). Briefly, bloodfed mosquitoes' abdomens were processed using Boom extraction method [10]. The Authentifiler™ PCR Amplification kit (Applied Biosystems®), with nine human microsatellite markers and one gender marker, was used to compare human DNA in blood meals and in blood samples collected from study participants. Capillary electrophoresis was used to determine DNA profiles.

Mosquito speciation assay and malaria parasite detection by PCR

DNA was extracted from individual head-thoraces using the DNAzol procedure (Invitrogen). Mosquito speciation was performed using a single PCR-RFLP assay as

described by Fanello and colleagues [11], and nested PCR [12] was used for *Plasmodium falciparum* detection.

Statistical analysis

Stata version 14 (Stata Corporation, College Station, TX, USA) was used for statistical analysis. Demographic data were not available for five houses where no bloodfed mosquitoes were collected; study participants living in these households are not included in the analyses presented here. We used the number of bloodfed *Anopheles* mosquitoes collected per day per household when comparing different surveys. Wilcoxon signed-rank test was used to assess within-household changes in *Anopheles* abundance. A mixed effects logistic model was used to quantify the association between mosquito falciparum infection status and mosquito species; the analysis was adjusted for survey time (fixed effect), and household of collection was used as random effect.

Mixed effects negative binomial models [13, 14] were used to assess overdispersion in exposure to mosquitoes, after adjustment for variability linked to age and survey time (fixed effects) and household-level variation (random effect). These models were also used in *Anopheles* species-specific analysis. Likelihood-ratio test compared mixed effects negative binomial models and mixed effects Poisson models.

Ethics

Ethical clearance was obtained from the London School of Hygiene & Tropical Medicine ethics committee (reference number 6447), and the ethics committee of the Centre National de Recherche et de Formation sur le Paludisme (Burkina Faso).

Results

Study households

Thirty-five households were included in this analysis. The median number of individuals living in each study household was 3 (range, 2 – 5). We collected 325, 620

and 190 bloodfed *Anopheles* mosquitoes at the start (2014), peak (2014) and end (2013) of the transmission season, respectively (**Table 1**). During the 2013 survey, 21/35 houses had at least one bloodfed mosquito collected; in 2014, 19/20 and 20/20 households had fed mosquitoes collected at the start and peak of transmission season, respectively. The average number of bloodfed mosquitoes collected per day in each household was higher at the start (median 2.3, interquartile range [IQR] 0.7 – 5.5) and peak (median 3.9, IQR 2.1 – 8.5) compared to the end (median 0.1, IQR 0 – 0.6 mosquitoes per day) of the transmission season ($P = 0.001$ and <0.001 , respectively). There was no correlation between the average number of bloodfed mosquitoes collected per day and the number of individuals living in each household (Spearman's rank correlation coefficients, 0.15, 0.03 and 0.06 for the first, second and third surveys respectively; all $P > 0.05$). Of note, at the end of 2013 transmission season, most mosquito collections performed after mid-November were unsuccessful, suggesting a village-wide reduction in mosquito abundance during this period (*Supporting Information Figure S1*).

Table 1. Study surveys.

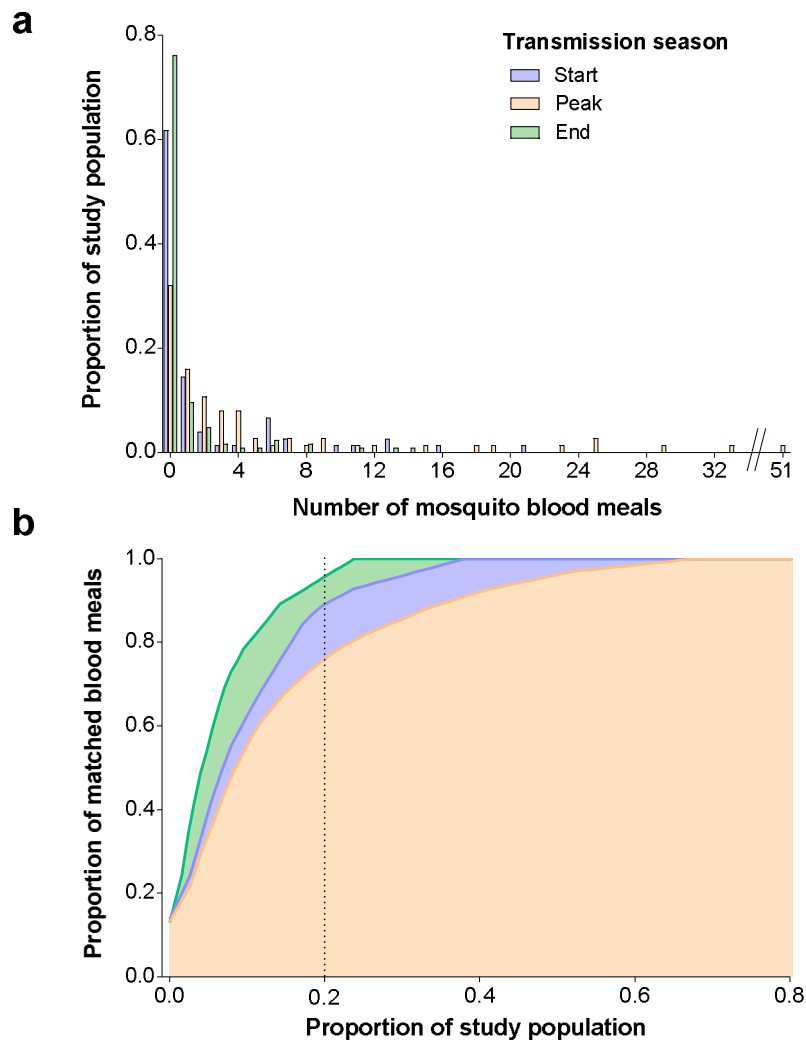
	First Survey	Second Survey	Third Survey
Start Date	October, 2013	June, 2014	September, 2014
Number of sampling days	54	20	20
Timing	End of transmission season	Start of transmission season	Peak of transmission season
Number of households	35*	20	20
Number of participants	126	81	77
	<i>N (%)</i>	<i>N (%)</i>	<i>N (%)</i>
Age categories**			
< 5 years	20 (15.9)	12 (14.8)	12 (15.6)
5 - 15 years	62 (49.2)	39 (48.2)	37 (48.0)
> 15 years	44 (34.9)	30 (37.0)	28 (36.4)
Gender			
Male	41 (32.5)	22 (27.2)	21 (27.3)
Female	85 (67.5)	59 (72.8)	56 (72.7)
Number of bloodfed mosquitoes collected	190	325	620

*Demographic information not available for individuals living in 5/40 households (first survey only); **Age at enrolment (first survey)

Heterogeneity in actual exposure to malaria vectors

Most (1,066/1,135) collected bloodfed mosquitoes had their blood meal analysed. 100 mosquitoes had no amplification or only few alleles amplified. More than 60% (666/966) of successfully typed blood meals were matched to single study participants living in the household where they were collected; this was consistent across surveys (65.2, 71.6 and 70.4% at the start, peak and end of transmission season, respectively). Mosquito blood meals with more than two alleles in at least three loci (N=153) were considered to have multiple human DNA sources and were not included in this analysis. In **Figure 1**, the distributions of the number of mosquito bites each individual received during different study surveys are presented. Approximately 20% of individuals, including children and adults, provided 85.1, 76.0 and 95.5% of mosquito blood meals at the start, peak and end of the transmission season, respectively. Throughout the study, a small number of individuals, mostly adults, were matched to considerably higher numbers of blood meals compared to the rest of the population. Conversely, 32.0 – 76.2% of study participants were not linked to bloodfed mosquitoes during the study surveys. In an analysis of data from all surveys, mixed effects negative binomial regression better explained the distribution of mosquito bite counts than mixed effects Poisson models ($P < 0.001$), after adjustments for age and survey and household-level data correlatedness. In sensitivity analyses that assign blood meals with multiple human DNA sources to the least exposed individuals in each household, the ~20% of the population with most mosquito bites were linked to at least 70% of matched blood meals (*Supporting Information Figure S2*).

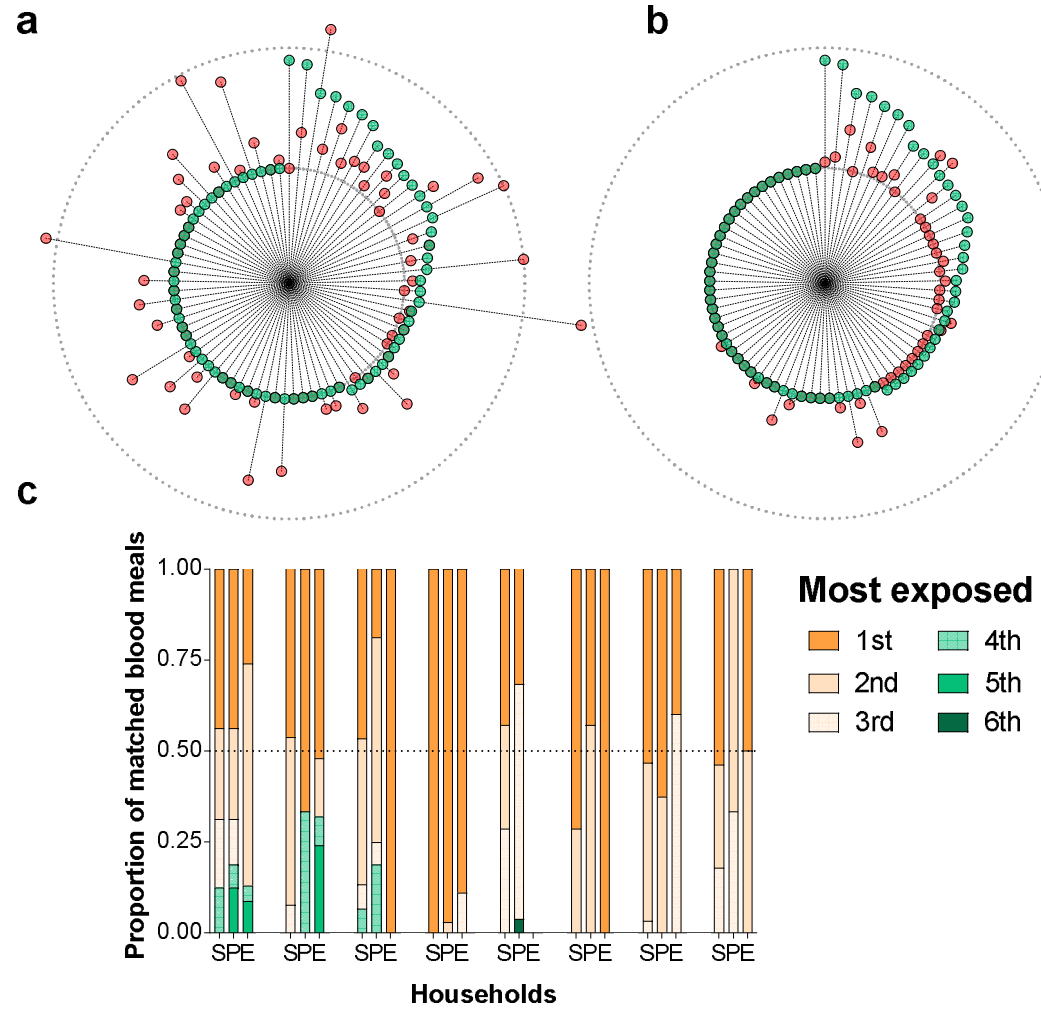
Figure 1. Inter-individual variation in exposure to *Anopheles* mosquitoes. In **a**, the distributions of mosquito blood meals matched to each study participant are presented for the three different surveys. In **b**, the cumulative proportion of mosquito blood meals (y-axis) matched to study participants (x-axis) sorted by number of mosquito bites received is presented for the different surveys. At the peak of the transmission season, six individuals were absent or only present during one collection day and were not included in this graph. Only singly matched bloodfed mosquitoes linked to individuals living in the same household where they were collected are included in this figure.



For individuals who participated in all surveys, there were positive correlations between numbers of matched mosquitoes 1) at the start and peak of transmission season (Spearman's rank correlation coefficient 0.24, $P=0.04$) and 2) at the start and end of transmission season (Spearman's rank correlation coefficient 0.33, $P=0.002$), suggesting some consistency in preferential biting. However, some individuals with highest numbers of matched blood meals at the start of the transmission season received few or no mosquito bites in other surveys (**Figure 2A and B**).

Within-household heterogeneity in mosquito exposure was observed: in many households, the most exposed individual was the source of more than 50% of mosquito blood meals (**Figure 2C**). While individuals with high numbers of matched blood meals often lived in households with high total numbers of matched mosquitoes (*Supporting Information Figure S3*), in all houses included in this study there were individuals with relatively low mosquito exposure.

Figure 2. Temporal (**a** and **b**) and within-household (**c**) variation in exposure to malaria vectors. In **a** and **b**, individuals were ranked according to the number of matched mosquito blood meals per collection day at the start of the transmission season; each green circle represents a study participant and the distance between a green circle and the centre of the panel is proportional to the log_e-transformed number of mosquito bites received per collection day (see *Supporting Information* for details). Each red circle corresponds to the number of singly matched blood meals at the peak (**a**) or end (**b**) of the transmission season for the individual represented by the radially aligned green circle. The dotted inner circle corresponds to no mosquito bites, and the dotted outer circle, to 5 bites per day. In **c**, each of the three columns corresponds to a different survey (S, Start of transmission season; P, Peak of transmission season; E, End of transmission season) for a select number of households. Individuals in the same household are denoted by different colours, which are consistent in the different surveys. The proportions of matched blood meals linked to each individual by household and survey are on the y-axis; only the 8 households with at least 5 matched mosquitoes at the start of the transmission season and three or more study participants are shown. The numbers of individuals living in the households included in panel **c** are (from left to right): 5, 5, 4, 4, 6, 5, 3 and 3.



Anopheles species-specific feeding choices

Mosquitoes collected during the study were genotyped for species identification. At the start of the transmission season, *Anopheles coluzzii* represented 44.7% (142/318) of all bloodfed mosquitoes, while at the peak and end of the transmission season most bloodfed mosquitoes were *Anopheles gambiae sensu strictu* (74.0 [450/608] and 53.7% [102/190], respectively) (*Supporting Information Figure S1*). Species-specific distributions of blood meals matched to study participants are presented in **Figure S4** (*Supporting Information*) and suggest that heterogeneity in exposure to anopheline mosquitoes occurs irrespective of vector species. Of note, mixed effects negative binomial models better fit the distribution of *Anopheles* species-specific mosquito bites compared to mixed effects Poisson models (see *Supporting Information*). Additionally, rates at which individuals were bitten by mosquitoes of different species were positively associated (*Supporting Information Figure S5*) (all $P < 0.01$ in mixed effects negative binomial models that included number of species-specific matched blood meals and age as covariates).

Transmission of parasites from mosquitoes to humans

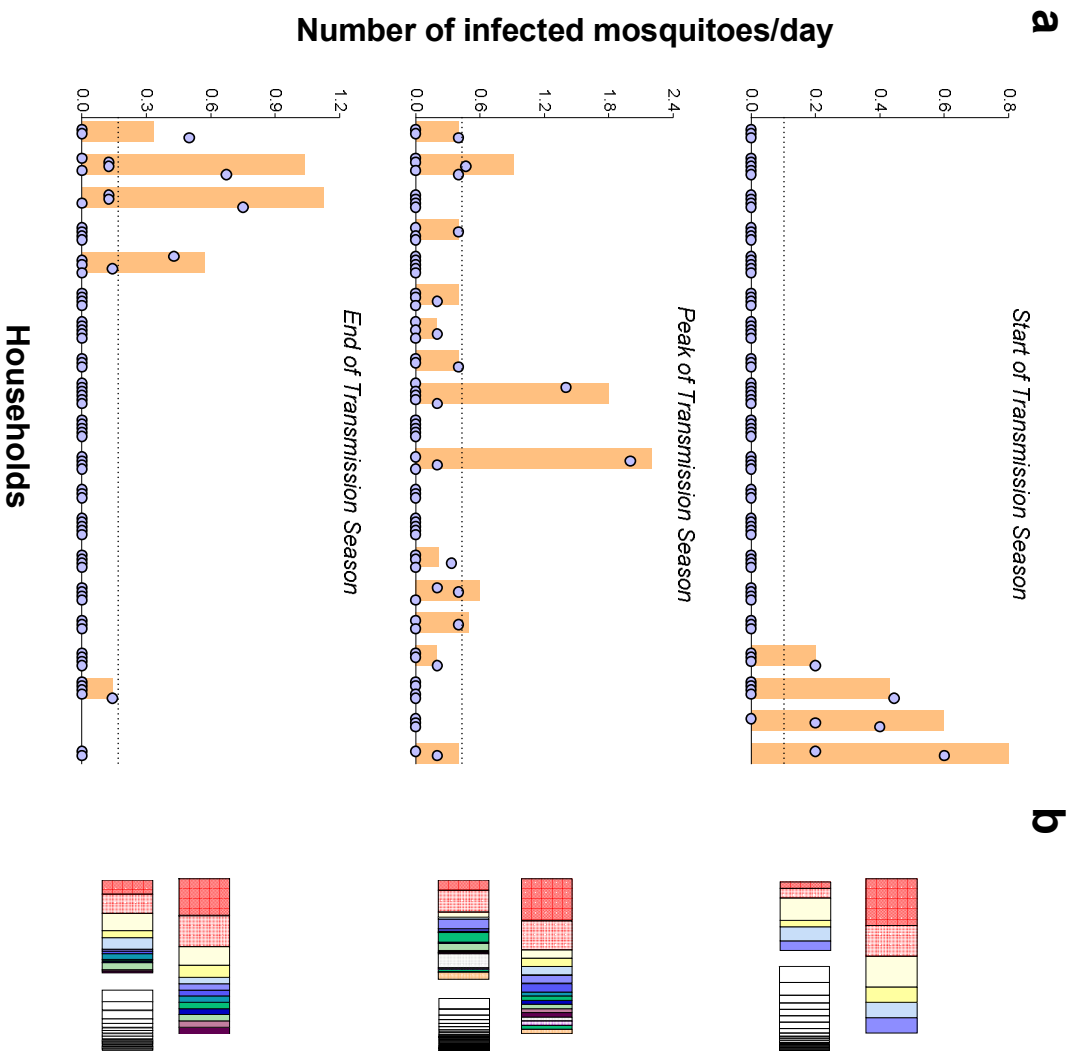
The prevalence of malaria parasites in bloodfed mosquitoes identified via PCR of head and thorax was higher at the end versus start and peak of the transmission season (22.8, 4.3 and 8.6%, respectively), and slightly higher in singly-matched mosquitoes compared to mosquitoes with multiple meal sources (*Supporting Information Table S1*). *A. gambiae s.s.* mosquitoes were more frequently infected compared to *A. coluzzii* ($P=0.002$) (**Table 2**). Individual- and household-level frequencies of exposure to infected mosquitoes are presented in **Figure 3A**. Only 7.7, 23.6 and 10.3% of study subjects were linked to at least one potentially infective blood meal at the start, peak and end of transmission season, respectively; the two individuals, equivalent to 1.6 – 2.8% of the study population at time of survey, with the highest numbers of matched meals from infected mosquitoes in each survey experienced 44 – 50% of all exposure to infected mosquitoes. As expected, there was a positive association between the number of potentially infective mosquito bites an individual received and the total number of matched meals regardless of mosquito parasitological status (**Figure 3B**, P

< 0.001 in a mixed effects negative binomial model that included total number of matched blood meals as covariate).

Table 2. Mixed effects logistic model on mosquito infection status

		Odds ratio (95% CI)	P-value
Mosquito species			
	<i>A. gambiae s.s.</i>	Reference	
	<i>A. coluzzii</i>	0.41 (0.23 – 0.72)	0.002
	<i>A. arabiensis</i>	0.55 (0.20 – 1.50)	0.24
Timing (Transmission Season)			
	<i>End</i>	Reference	
	<i>Start</i>	0.18 (0.09 – 0.39)	<0.001
	<i>Peak</i>	0.29 (0.16 – 0.53)	<0.001

Figure 3. Rates of exposure to potentially infective mosquito bites (i.e. mosquitoes with malaria infection detected by PCR) (**a**) and their association with total mosquito exposure (**b**). In **a**, blue circles represent study participants; individuals living in the same study house are presented together (orange bars). Y-axes show 1) the average numbers of falciparum-positive mosquito bites per individual per day (blue circles) or 2) the total numbers of falciparum-positive fed mosquitoes collected in each house per collection day (orange bars); y-axes' limits vary to improve visualization. Horizontal dotted lines represent the average, over all study houses, number of falciparum-positive bloodfed *Anopheles* mosquitoes collected per house and day. Only the 20 houses included in all three surveys are represented in this figure. In the three graphs, houses were ordered according to the number of fed mosquitoes collected at the start of the transmission season. In **b**, the top bar of each pair of horizontal bars presents the proportions of blood meals from infected mosquitoes linked to different individuals, represented by different colours, in each survey. The bottom bar of each pair corresponds to the proportions of all singly matched mosquito blood meals linked to different study participants; for each survey, each study participant is represented by the same colour for both infected and all mosquitoes. Individuals who were not matched to infected mosquitoes' blood meals are represented by white segments.



Discussion

In this study, we quantified natural exposure to *Anopheles* mosquitoes likely to be responsible for malaria transmission events having successfully fed on humans. We observed significant differences in the numbers of bloodfed mosquitoes matched to study participants that are consistent with the Pareto rule, with ~20% of participants being the source of more than 80% of all singly-matched blood meals. This heterogeneity was also apparent within-household, where individuals who provided most blood meals often contributed more than 50% of anopheline meals. Exposure to potentially infective mosquito bites also followed an aggregated pattern: 5.1, 13.9 and 6.3% of the population experienced 80% of parasite inoculations at the start, peak and end of the transmission season, respectively. Taken together, our observations indicate that relatively few individuals, not necessarily the same during the entire rainy season, might be responsible for driving malaria transmission by being repeatedly sampled and infected by malaria vectors. These data provide further insights into the mechanisms that lead to heterogeneity in human malaria infection risk [15-18].

A variety of studies have quantified inter-individual differences in attractiveness and exposure to mosquitoes. While studies involving experimental huts [1] and olfactometers [19] demonstrated the influence of individual-level factors such as pregnancy [20] and infection status [21] on attractiveness to mosquitoes, community-wide assessments of wild-caught mosquitoes [7, 22, 23] are necessary to quantify variation in exposure to malaria vectors over larger scales. A study in The Gambia [7] used ABO group and haptoglobin typing to identify the sources of mosquito blood meals; the small number of variants in these markers [24] limited the selection of households. In Tanzania [22], microsatellites were used to link blood meals to humans to assess the protection afforded by bed nets against mosquitoes; a high proportion of the ~250 analysed bloodfed mosquitoes were matched to individuals sleeping in the same room where they were collected, including in the village with bed nets, and 80% of matched blood meals came from less than 20% of the population. In our study, we matched 666 blood meals to individual study participants and observed that the distribution of mosquito bites was overdispersed, with 76.0 – 95.5% of singly matched blood meals originating from ~20% of the study population. These results corroborate the findings of the study in Tanzania and of a different study in western Kenya [25],

where 16% of the study participants were matched to 58% of *Anopheles* blood meals, but show even more unequal exposure patterns across three timepoints in the season. The fact that the majority of human-mosquito encounters in our study area involved only a few individuals is a consequence of both between- and within-household variation in exposure. Indeed, at the peak of the transmission season, the household with the highest number of fed mosquitoes had ~150 times more than the household with the lowest number. Household characteristics, such as construction material, number of windows, eaves as well as geographical proximity to mosquito breeding sites have all been associated with increased exposure to mosquitoes indoors and explain some of the between-household variation in mosquito abundance. We found that household heterogeneity is compounded by inter-individual variation in frequency of sampling by mosquitoes for people living in the same house. At the peak of the transmission season, the maximum difference in numbers of matched meals for participants in the same house ranged from 1 to 53, i.e. a difference of up to ~10 mosquito bites per day. Age and body size can partially explain differences in attractiveness [4] yet we noted age-matched individuals in the same house often with dissimilar mosquito exposure (*Supporting Information Figure S6*). Other individual-level characteristics such as odours [19, 26], (effective) use of protective measures or behaviour will be relevant in natural settings. Of note, the age distribution of our study population (**Table 1**), that included more children than adults, might not reflect the true demography of the region. Although our results are in agreement with previous studies, we cannot exclude that a difference in age composition, possibly related to the fact that only houses with at least one child were included, could have influenced our heterogeneity estimates. A technical limitation of this study was that we were not able to unequivocally match multiple source blood meals; however, in a conservative sensitivity analysis that assigned these meals to the individuals with lowest exposure, most mosquito bites were still linked to a small proportion of the population.

Broad differences in anthropophily between mosquito species are well recognised [27] however there are less data [28] on whether different *Anopheles* species might have different feeding preferences with regards to individual humans, or whether some individuals might be attractive to a particular mosquito species but not to others. In this study, we analysed blood meals in mosquitoes from three species: *A. gambiae* s.s., *A. coluzzii* and *Anopheles arabiensis*. Although seasonal differences in species

abundance were evident, we observed aggregation in human biting irrespective of vector species and exposure to one species was positively associated with exposure to the others. This suggests heterogeneity in exposure to anopheline mosquitoes is a common epidemiological phenomenon including in areas with different vector populations.

We also determined how the frequency of host-vector contacts might influence the variation in the incidence of falciparum infections in human populations. Between 75 and 90% of the study participants were not matched to feeding by infected mosquitoes during the three study periods. Whilst this does not represent all infected mosquito bites these individuals received, it does highlight the degree of heterogeneity in biting. Some study participants received multiple potentially infective bites over a period of a few days; whether these individuals developed liver- and blood-stage infections and eventually symptoms and how different inoculations and ongoing blood-stage infections interfered with each other [29] is not known. Studies that combine mosquito blood meal typing and parasitological and clinical follow-up of human populations would be informative to understand heterogeneity in clinical malaria risk [30-32].

In summary, although studies have assessed natural exposure to vectors of other infections, such as *Aedes* [33, 34] and *Culex* [35], only limited data are available for *Anopheles* mosquitoes [22, 25]. In our field site, characterized by high malaria transmission intensity, we show significant heterogeneity both between and within households in terms of the number of mosquito blood meals and the distribution of potentially infective mosquito feedings; these patterns are consistent with the 20/80 rule and support the design of interventions that aim to reduce transmission by targeting a small proportion of the population. A quantitative understanding of the processes leading to this heterogeneity in mosquito exposure would inform at which level such interventions should be targeted, household or individual; this would require quantification of the relative contributions of household-level factors, differential attractiveness to mosquitoes and human behavioural factors.

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Supporting Information

Title: Variation in natural exposure to anopheles mosquitoes and its effects on malaria transmission

Visualisation of temporal variation in actual exposure to malaria vectors

In **Figure 2**, individuals were ranked according to the number of matched mosquito blood meals per collection day. The distance between each observation and a central point is proportional to the log-number of mosquito bites per collection day. Since several individuals were not matched to any mosquito blood meal, the mathematical constant e was added to each observation to allow inclusion of all data in panels **a** and **b**. Polar coordinates were used, with the radii equal to $\log_e(\text{number of mosquito bites per collection day} + e)$ and the angle varying with the ranking at the start of the wet season.

Anopheles species-specific count models

Mixed effects negative binomial models that had as dependent variables the numbers of *coluzzii* and *A. gambiae* s.s. blood meals were fit. In these models, the conditional overdispersion parameter was significantly different from zero: 1.9 (95% CI, 1.2 – 3.2) and 2.5 (95% CI, 1.8 – 3.6) for models of *A. coluzzii* and *A. gambiae* s.s. mosquito bites, respectively.

Figure S1. Distribution of bloodfed mosquitoes by species and collection day.

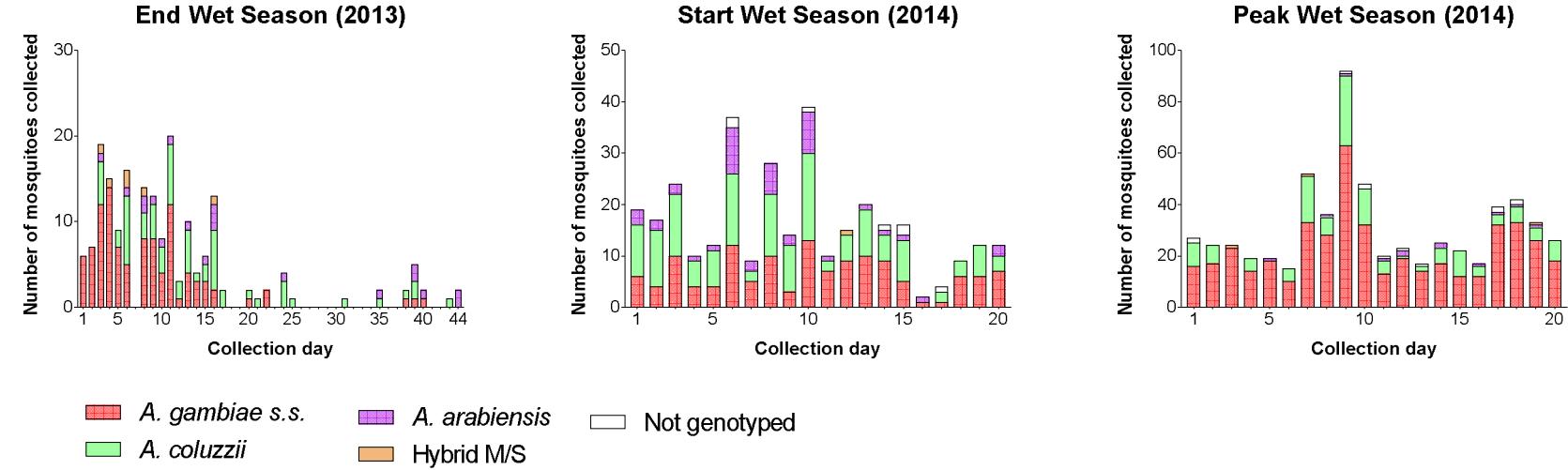


Figure S2. Sensitivity analyses. In **Figure 1**, only singly-matched mosquitoes were included. A total of 153 mosquito blood meals were likely to have at least two human DNA sources. In this graph, two different scenarios were assumed: in Scenario 1, each mosquito blood meal with multiple sources was assigned to the two individuals with most singly-matched meals in each household; in Scenario 2, each blood meal with multiple human DNA sources was assigned to the two individuals with lowest numbers of singly-matched mosquito meals in each household. The cumulative proportion of blood meals (y-axis) matched to study participants (x-axis) sorted by number of mosquito bites received is presented for the three different surveys and two different scenarios. Only individuals present on at least 3 mosquito collection days were included in this graph.

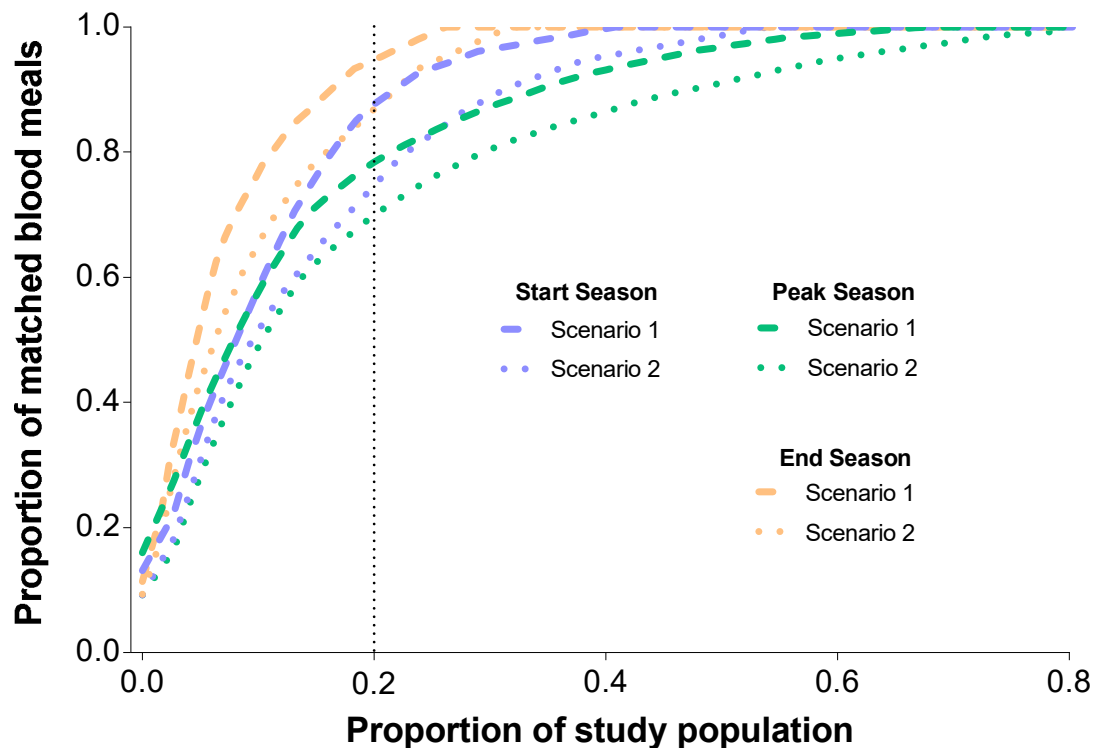


Figure S3. Relationship between household- and individual-level exposures to *Anopheles* mosquitoes. The y-axis represents the number of mosquito blood meals linked to each study participant (left column) and the total number of blood meals matched to individuals living in each study household (right column). In each panel, individuals living in the same household are represented by circles of the same colour and linked to their household. Only houses with at least 10 (start and peak of transmission season) or 5 (end of transmission season) matched mosquitoes are included in this graph; in all other houses, all individuals had 5 mosquito bites or fewer at the start or peak of the wet season, and 3 or fewer bites at the end of the transmission season.

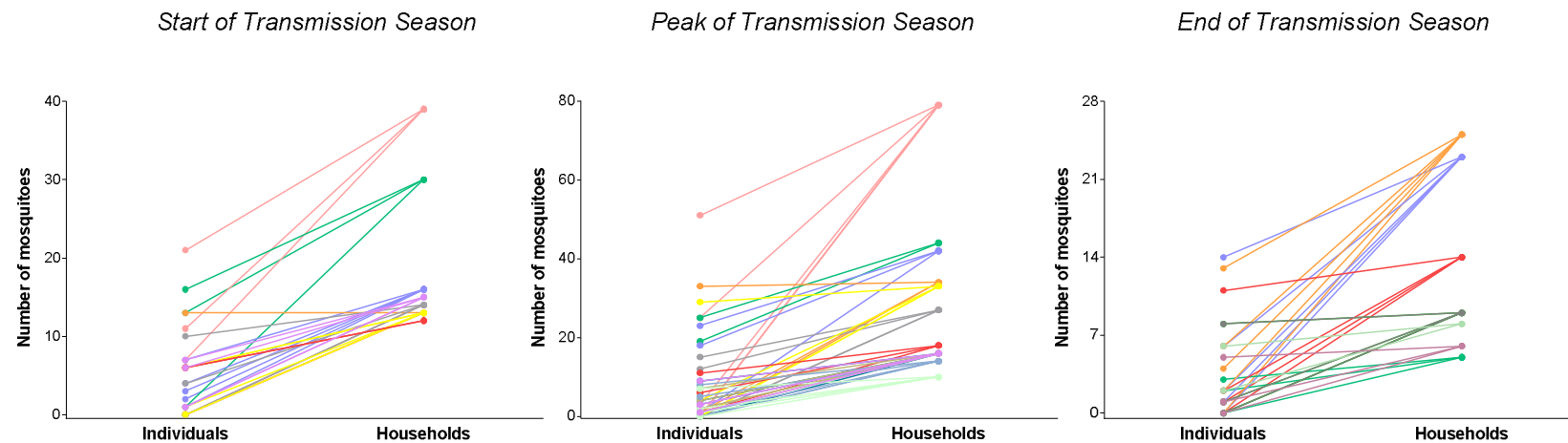


Figure S4. Cumulative proportion of *Anopheles* species-specific blood meals (y-axes) matched to study participants (x-axes) sorted by number of mosquito bites received. Only individuals present in at least 3 mosquito collection days were included in this graph. **a**, *A. gambiae* s.s.; **b**, *A. coluzzii*. Only a few (7 – 16) *A. arabiensis* mosquitoes were matched to study participants.

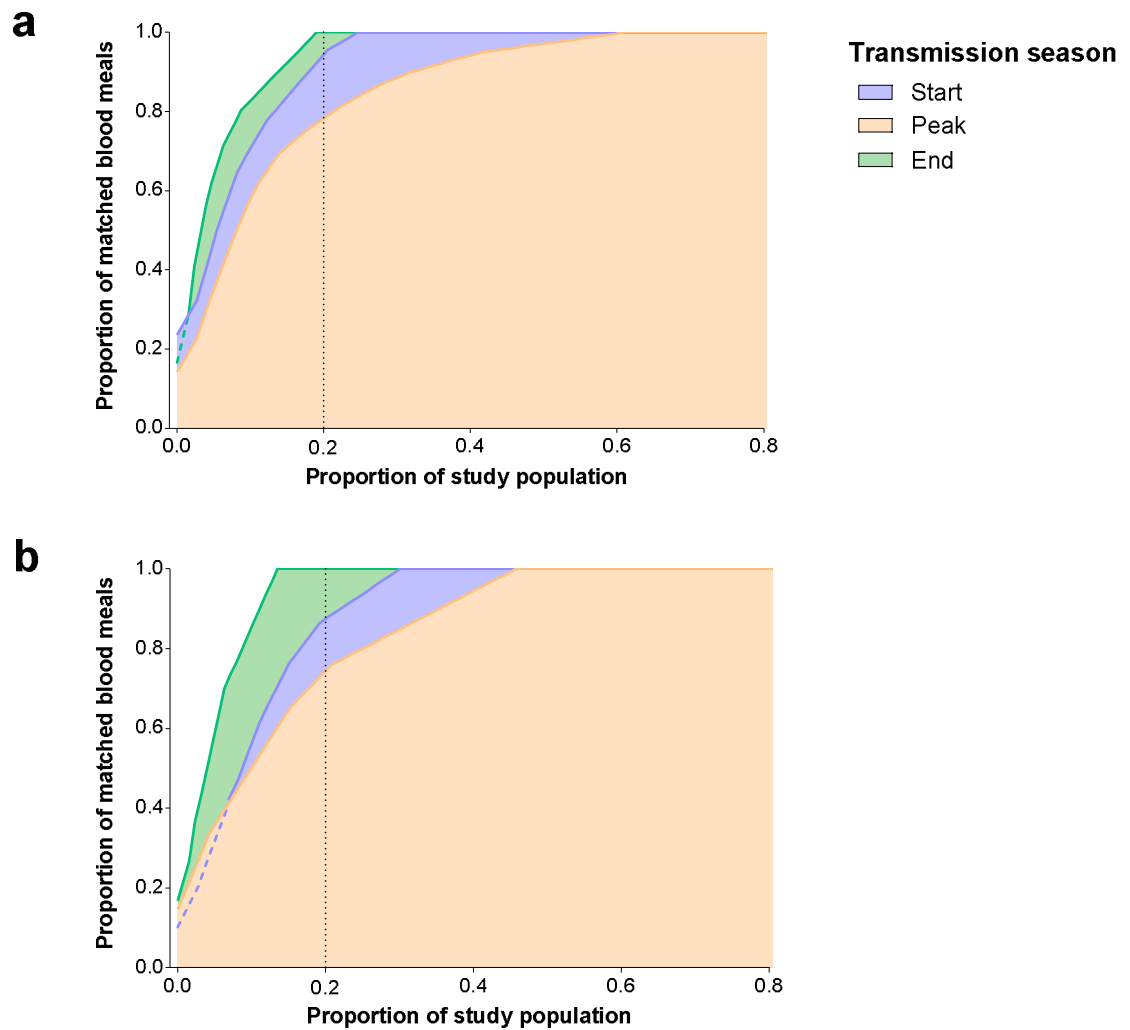


Figure S5. Correlations between vector species-specific numbers of matched blood meals. Both y- and x-axes represent the number of mosquito blood meals matched to study participants; each circle corresponds to a study participant. Axes limits vary to allow better visualisation of patterns in different surveys.

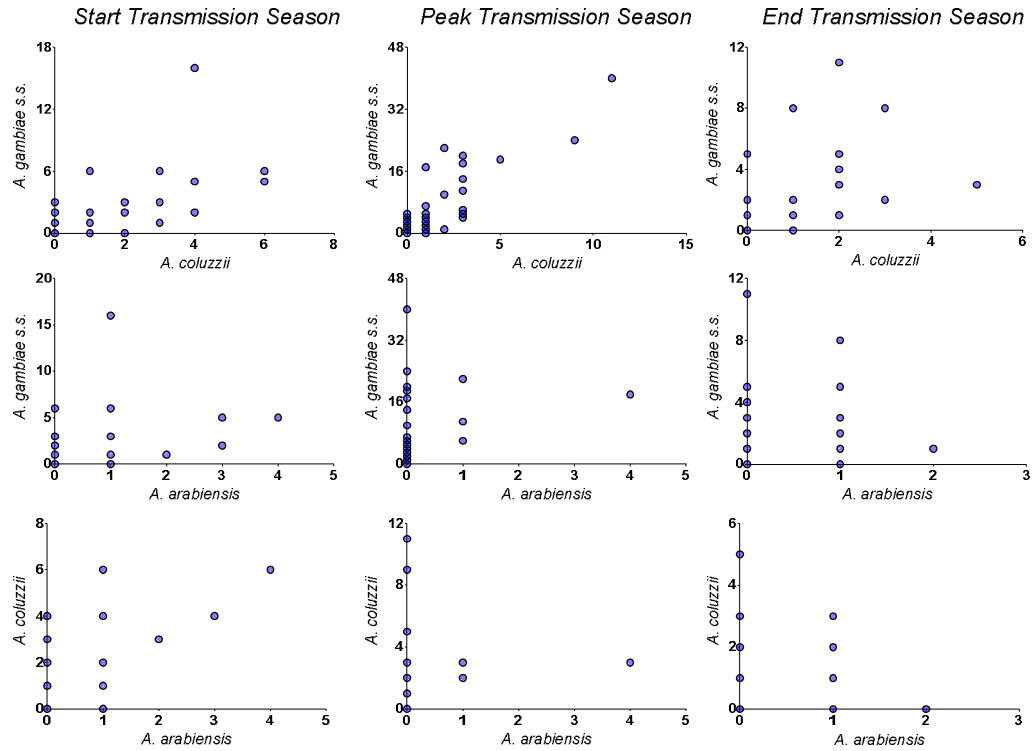


Figure S6. Age-independent variability in exposure to *Anopheles* mosquitoes. Individuals whose age difference was 2 years or less and who lived in the same household are presented. In the first household, two pairs of age-matched individuals were included. For each pair, the youngest individual is presented on the left. Numbers of mosquito bites in different surveys are represented by different colours.

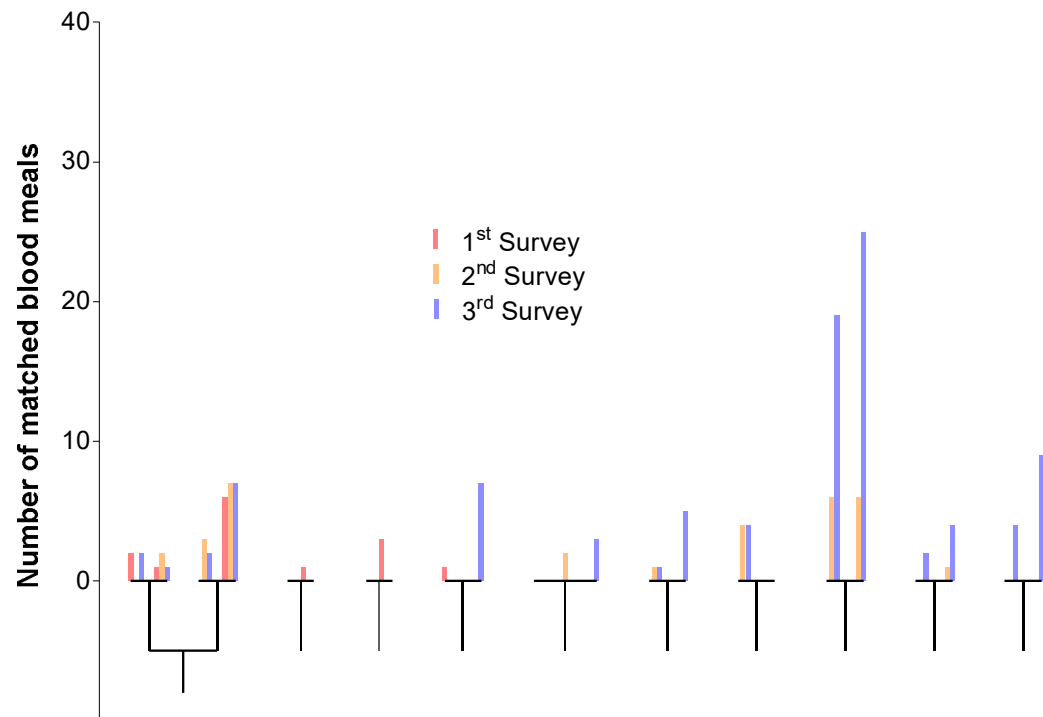
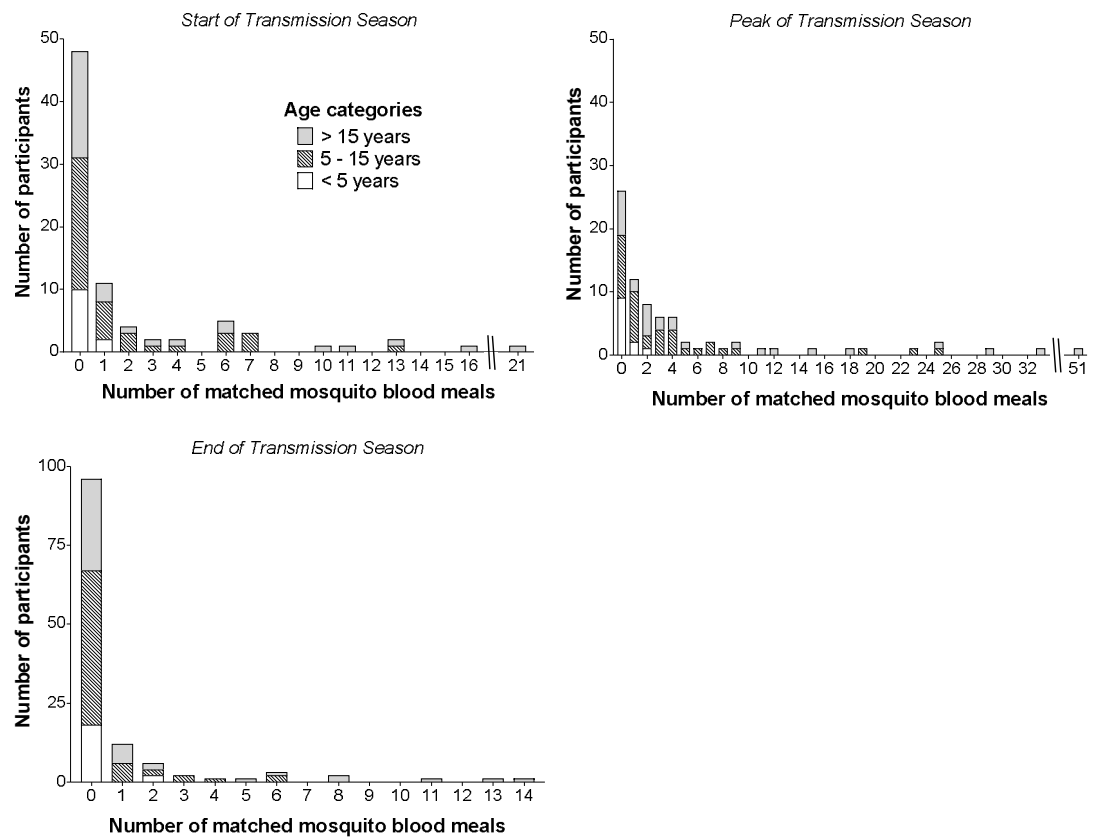


Table S1. Prevalence of mosquito infection, determined by nested PCR performed using mosquitoes' head-thoraces, by number of mosquito blood meal sources. Only mosquitoes with both blood meal typing and infection information were included in this table.

Prevalence of mosquito infection		
	<i>Singly-matched, % (n/N)</i>	<i>Multiple human source, % (n/N)</i>
Timing (Transmission Season)		
<i>Start</i>	6.2 (10/160)	3.3 (3/90)
<i>Peak</i>	9.9 (37/375)	0 (0/40)
<i>End</i>	23.1 (25/108)	21.1 (4/19)

Additional Figure

Additional Figure 1. Distribution of matched mosquito blood meals (x-axes) by survey and age.



Chapter 5, Primaquine to block malaria transmission

The epidemiological and entomological studies described in **Chapters 3** and **4** provided answers to questions such as “Who are the individuals likely to infect mosquitoes in natural settings?”. The work presented in this chapter aimed to assess the efficacy of primaquine, a drug that is routinely used in vivax malaria treatment and is now recommended to block malaria transmission, in reducing post-treatment infectiousness of individuals receiving first-line antimalarial therapy. This study was published in *BMC Medicine* in 2016 (<https://doi.org/10.1186/s12916-016-0581-y>) and the version accepted for publication is included here. An additional figure is included at the end of the chapter.



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Student	Bronner Pamplona Augusto Gonçalves
Principal Supervisor	Chris Drakeley
Thesis Title	The human infectious reservoir of falciparum malaria

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Where was the work published?	BMC Medicine		
When was the work published?	8 March 2016		
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Student Signature: Bronner P. A. G. Date: 04/09/2017

Supervisor Signature: Chris Drakeley Date: 04/09/2017

Title page

Title: Single Low Dose Primaquine to Reduce Gametocyte Carriage and *Plasmodium falciparum* Transmission after Artemether-Lumefantrine in Children with Asymptomatic Infection: A Randomised, Double-Blind, Placebo-Controlled Trial

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Running title: Primaquine and malaria transmission

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Abstract

Background

A single low dose (0.25 mg/kg) of primaquine is recommended as a gametocytocide in combination with artemisinin-based combination therapies for *Plasmodium falciparum* but its effect on post-treatment gametocyte circulation and infectiousness to mosquitoes has not been quantified.

Methods

In this randomised, double-blind, placebo-controlled trial, 360 asymptomatic parasitaemic children aged 2-15 years were enrolled and assigned to receive: artemether-lumefantrine (AL) and a dose of placebo; AL and a 0.25 mg/kg primaquine dose; or AL and a 0.40 mg/kg primaquine dose. On days 0, 2, 3, 7, 10 and 14, gametocytes were detected and quantified by microscopy, Pfs25 mRNA quantitative nucleic acid sequence based amplification (QT-NASBA), and quantitative reverse-transcriptase PCR (qRT-PCR). For a subset of participants, pre- and post-treatment infectiousness was assessed by mosquito feeding assays on days -1, 3, 7, 10 and 14.

Results

Both primaquine arms had lower gametocyte prevalences after day 3 compared to the placebo arm, regardless of gametocyte detection method. The mean (95% confidence interval) number of days to gametocyte clearance in children with patent gametocytes on day 0 (N=150) was 19.7 (14.6 – 24.8), 7.7 (6.3 – 9.1) and 8.2 (6.7 – 9.6) for the AL-placebo, the 0.25 mg/kg primaquine dose and the 0.40 mg/kg primaquine dose arms, respectively. While 38.0% (30/79) of selected gametocytaemic individuals were infectious before treatment, only 1/251 participant, from the AL-placebo group, infected mosquitoes after treatment.

Conclusions

We observed similar gametocyte clearance rates after 0.25 and 0.40 mg/kg primaquine doses. Infectivity to mosquitoes after AL was very low and absent in primaquine arms.

ClinicalTrials.gov Registration. NCT01935882

Background

Infectiousness to malaria vectors depends on the presence of sexual stage parasites, gametocytes, in the peripheral blood and is essential to sustain transmission in endemic areas [1]. Shortening the duration of gametocyte circulation reduces the probability of parasite spread and might, therefore, facilitate control. Artemisinin-based combination therapy (ACT) is universally adopted as first-line treatment for clinical falciparum malaria [2], effectively clears asexual parasites and immature gametocytes, and is associated with lower post-treatment infectivity based on membrane feeding assays compared to other antimalarials [3]. Mature gametocytes, however, persist after ACT in microscopic or submicroscopic concentrations and residual transmission has been reported following sulphadoxine-pyrimethamine in combination with artesunate [4, 5], dihydroartemisinin-piperaquine [4, 6] and artemether-lumefantrine (AL) [4, 6].

Primaquine, tafenoquine and methylene blue radically clear mature gametocytes; primaquine being the only commonly used as antimalarial [7]. The World Health Organization recommends a single low dose (0.25 mg/kg) as a gametocytocide in combination with ACT for *Plasmodium falciparum* malaria in elimination and artemisinin resistance containment scenarios [8]. However wide-scale adoption of this recommendation is limited by safety concerns [9], despite evidence that a higher dose, 0.40 mg/kg, is associated with only minor haematological changes in G6PD normal children [10]. Furthermore, the effect of the 0.25 mg/kg dose in clearing gametocytes or preventing transmission after currently used ACT regimens has not been formally assessed. A dose-finding study in Uganda suggested a 0.40 mg/kg dose is as efficacious in clearing gametocytes as the previously recommended dose of 0.75 mg/kg [10], which has been associated with haemolysis [11]. At present, only mosquito feeding assays can truly determine the lowest efficacious dose of primaquine for preventing transmission [12].

Here, we compare gametocyte dynamics after AL alone or in combination with 0.25 mg/kg or 0.40 mg/kg primaquine dose in children with asymptomatic falciparum infection. We selected asymptomatic parasite carriers since these contribute considerably to the infectious reservoir for malaria [13, 14] and malaria elimination

and artemisinin containment strategies will need to involve the targeting of asymptomatic malaria infections [15]. For a subset of participants, post-treatment infectiousness was determined by mosquito membrane feeding experiments.

Methods

Role of the funding source

Authors had full access to the trial data. Funders had no role in study design, data collection, analysis, interpretation and decision to publish study findings.

Study design

For this randomised, double-blind, placebo-controlled trial, participants were recruited between September 2013 and October 2014 from Balonghin, district of Saponé, an area with seasonal malaria in Burkina Faso [16].

Eligible individuals were asymptomatic children aged 2 to 15 years, weighing 10 kilos or more, with normal glucose-6-phosphate dehydrogenase (G6PD) activity based on BinaxNOW rapid diagnostic test (Alere Inc., Massachusetts, United States) and carrying patent *P. falciparum* asexual parasites or gametocytes. Initially, individuals were recruited regardless of microscopy gametocyte status (Study Phase A). Since no mosquito infections were detected (0/106 participants), the parasitological criterion for inclusion in infectiousness assessments was modified to include only children with patent gametocytes at screening, regardless of their asexual parasite count, to maximize pre-treatment infectivity (Study Phase B). Exclusion criteria were: haemoglobin level at screening lower than 8 g/dL, fever or history of fever in the last 24 hours, evidence of severe illness, known allergy to study medications, antimalarials taken in the last 48 hours, primaquine use in the last 4 weeks, blood transfusion in the last 90 days, and non-falciparum malaria infection at screening. Overall, 360 children were enrolled: 210 in Study Phase A (inclusion criterion: asexual parasite density 1,000 - 200,000 parasites/ μ L) and 150 participants in Study Phase B (inclusion criterion: presence of gametocytes by microscopy) (**Figure 1**).

Ethics, consent and permissions

This clinical trial was conducted in accordance with Good Clinical Practice guidelines, and was registered with ClinicalTrials.gov (number NCT01935882). Ethical clearance was obtained from the London School of Hygiene and Tropical Medicine ethics committee (reference number 6274), and the Comité d'Ethique pour la Recherche en Santé (Ministère de la Santé du Burkina Faso; reference number 2012-10-78). Written informed consent was obtained from parents or guardians, and assent, from children aged 12 years or older. The original protocol for the clinical trial (**Additional file 2**) and the supporting CONSORT checklist (**Additional file 3**) are provided as supporting information.

Randomisation and masking

Children were enrolled by the study clinician and subsequently randomly allocated by the study pharmacist, with equal probabilities, to three treatment arms: (1) AL as standard 6-dose regimen in combination with a single dose of placebo; (2) AL and a single 0.25 mg/kg primaquine dose; and (3) AL and a single 0.40 mg/kg primaquine dose.

Treatment assignment was stratified by gender, and sealed envelopes in block sizes of six (three study arms and two membrane feeding assays schedules [days 3 and 10 or 7 and 14 after treatment initiation]) were used. After the first 143 children were recruited, mosquito feeding experiments were amended: gametocytaemic children were randomised to have feeding assays on days -1 (one day before treatment initiation) and 7 or 3 and 7.

Treatment concealment was achieved by the addition of a syrup, which masked colour and taste of primaquine and placebo. Participants, investigators, and staff who were not involved in study drug administration were blinded to study arm allocation.

Procedures

AL (Coartem®; Novartis Pharma, Basel, Switzerland) was administered as half a tablet (20 mg of artemether and 120 mg of lumefantrine) per 5 kg of body weight in a 6-dose regimen over three days. Primaquine doses were prepared as previously described [10] and given with the 5th dose (day 2) of AL. All treatment doses were administered under supervision and with fatty food (biscuits) to ensure adequate absorption [17] and minimise the risk of gastro-intestinal side effects. If a child vomited within 30 minutes after a study medication was given, treatment was re-administered.

Participants were asked to return to the study clinic on days 0, 1, 2, 3, 7, 10 and 14. On day 0 (day of enrollment), AL administration was initiated. On each follow-up day, study subjects were examined and, except on day 1, had a blood slide prepared to detect malaria parasites and their haemoglobin levels quantified by Hemocue photometer (HemoCue AB, Angelholm, Sweden). Smears were screened for asexual stage parasites and gametocytes, and double-read. Biochemistry and full blood count assessments were performed on days 0, 3 and 7 on venous samples.

50 µL-finger-prick-blood samples were collected on all visits, except on day 1, stored in 250 µL of RNAprotect® cell reagent (Qiagen), and used for gametocyte detection and quantification: quantitative nucleic acid sequence based amplification (QT-NASBA) [18] was performed for samples collected on days 0 and 7; and quantitative reverse-transcriptase PCR (qRT-PCR), which allows better quantification [19], was used to estimate gametocyte densities in all available samples for individuals with patent gametocytes on day 0. Briefly, total nucleic acids (NA) were extracted using a MagNAPure LC automatic extractor (Total Nucleic Acid Isolation Kit – High Performance, Roche Applied Science). QT-NASBA was performed directly on total NA samples as described [18], with a minor change in the KCl concentration used: 60 instead of 80 mM. For gametocyte quantification by qRT-PCR, firstly remaining genomic human and parasite DNA were removed with the RQ1 DNaseI Digest Kit (Promega), then cDNA was synthesized with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems); from cDNA samples, Pfs25 sequence was amplified using primers described by Wampfler *et al* [19], GoTaq® qPCR Master Mix (Promega), and CFX96™ Real-Time PCR Detection System (BIO-RAD), using the standard cycling program as described in the manufacturer's instructions.

Amplification was set to continue for 40 cycles and cut-off for positivity was set at assigned gametocyte density of more than 0.02 gametocytes per μL , determined by a dilution series of mature stage V *in vitro* produced gametocytes (NF54 strain).

A subset of recruited children was invited to participate in membrane feeding assays [20] at two time-points (**Figure 1**), with the number of participants being dictated by the availability of mosquitoes. For each experiment, venous blood was taken and offered to 60 or more locally-reared female *Anopheles gambiae* mosquitoes. Fully fed mosquitoes were selected, kept on glucose at 27–29°C and dissected 7 days later. Midguts were examined for the presence of oocysts.

Primary and secondary endpoints

The primary efficacy endpoint was gametocyte clearance time (i.e., number of days to undetectable gametocyte levels) in the 0.25 mg/kg primaquine arm compared to the 0.40 mg/kg primaquine arm. The primary safety endpoint was maximal fall in haemoglobin levels during follow-up. Secondary efficacy endpoints were: gametocyte prevalences on days 3, 7, 10 and 14, and proportion of mosquitoes developing infection and their oocyst counts in feeding assays performed after treatment administration. Secondary safety endpoints included: number of participants requiring blood transfusion, maximal percentage decrease in haemoglobin concentration, proportion of participants with haemoglobin levels below 5 g/dL and number of severe adverse events.

Sample size

Sample size calculations were based on non-inferiority of 0.25 mg/kg versus 0.40 mg/kg primaquine dose. For the gametocyte clearance endpoint, the non-inferiority margin was 2.5 days [21]. If mean clearance time was 6.3 days [10] in both arms, with a standard deviation of 3, then 40 subjects per arm would give over 90% power at the one sided 0.025 level. For QT-NASBA-determined gametocyte prevalence on day 7, the non-inferiority margin was 12%. If prevalence in both arms was 10.6% [10], then 120 subjects per arm would give over 80% power at the one sided 0.025 level. The

number of feeding experiments and mosquitoes used in each assay were limited by logistical considerations and were not based on sample size calculations.

Statistical analysis

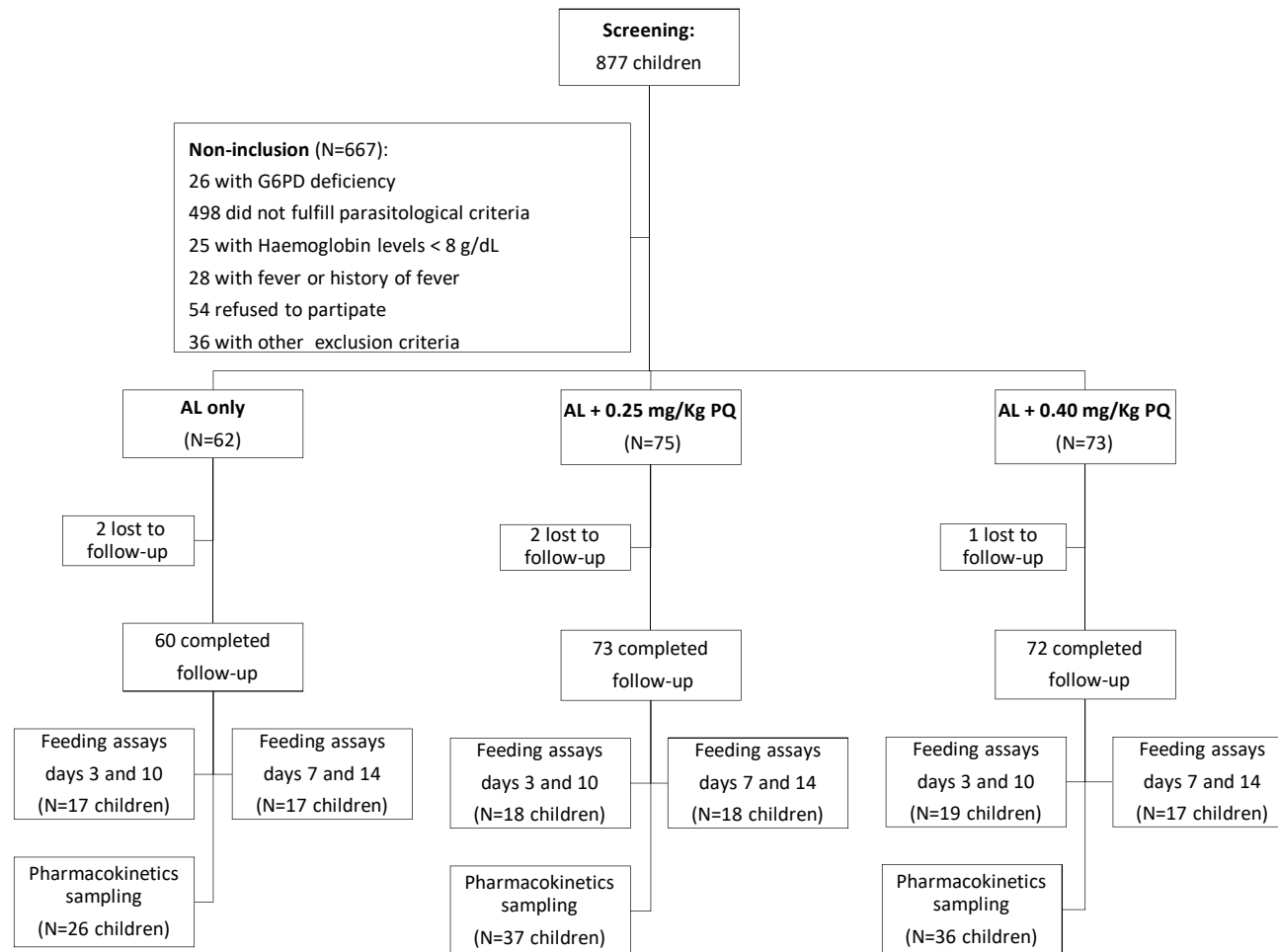
Stata 12.0 (Stata Corporation, Texas, United States) and SAS 9.3 (SAS Institute, North Carolina, United States) were used for statistical analysis. The mean time to gametocyte clearance was estimated for each treatment arm using a non-linear model [21] (**Additional file 1**); qRT-PCR data from children with patent gametocytes on day 0 were used (N=150). For the 0.25 mg/kg primaquine dose, a 95% confidence interval (CI) for the difference with the 0.40 mg/kg dose was calculated. The non-linear model was also used to assess superiority of the primaquine arms over the placebo group. Gametocyte prevalences on different visit days were compared between study arms by χ^2 or Fisher's exact test. Non-parametric tests (Mann-Whitney and Kruskal-Wallis) were used to compare parasite levels among different study groups. To quantify the effect of primaquine on transmission, post-treatment mosquito infection rates were compared between treatment arms. The maximal fall in haemoglobin levels measured by Hemocue photometer was presented as mean (95% CI) per study arm; Student's t-test was used for pair-wise comparisons.

Results

We screened 2,176 (877 in Study Phase A and 1,299 in Study Phase B) and enrolled 360 children (**Figure 1**). Twenty participants were enrolled despite having < 1,000 asexual parasites/ μ L and therefore not fulfilling all enrollment criteria but were retained in the analysis (**Additional file 1**). The median (interquartile range [IQR]) age at enrollment was 8 (5-10) years (**Table 1**). Median (IQR) asexual stage parasites concentration at enrollment was 1,824.5 (520–5,041) parasites/ μ L and similar among individuals receiving different treatment regimens.

Figure 1. Clinical Trial profile. (A) Study Phase where the presence of asexual parasites (1,000 - 200,000 parasites/ μ l) at screening was an inclusion criterion (initial 143 and final 67 participants). (B) Study Phase where children with patent gametocytes, regardless of their asexual parasite count, at screening were eligible (150 participants). Participants were considered to have a complete follow-up, if they had a total of 7 follow-up visits (days 0, 1, 2, 3, 7, 10, 14). AL = artemether-lumefantrine; PQ = primaquine.

A



B

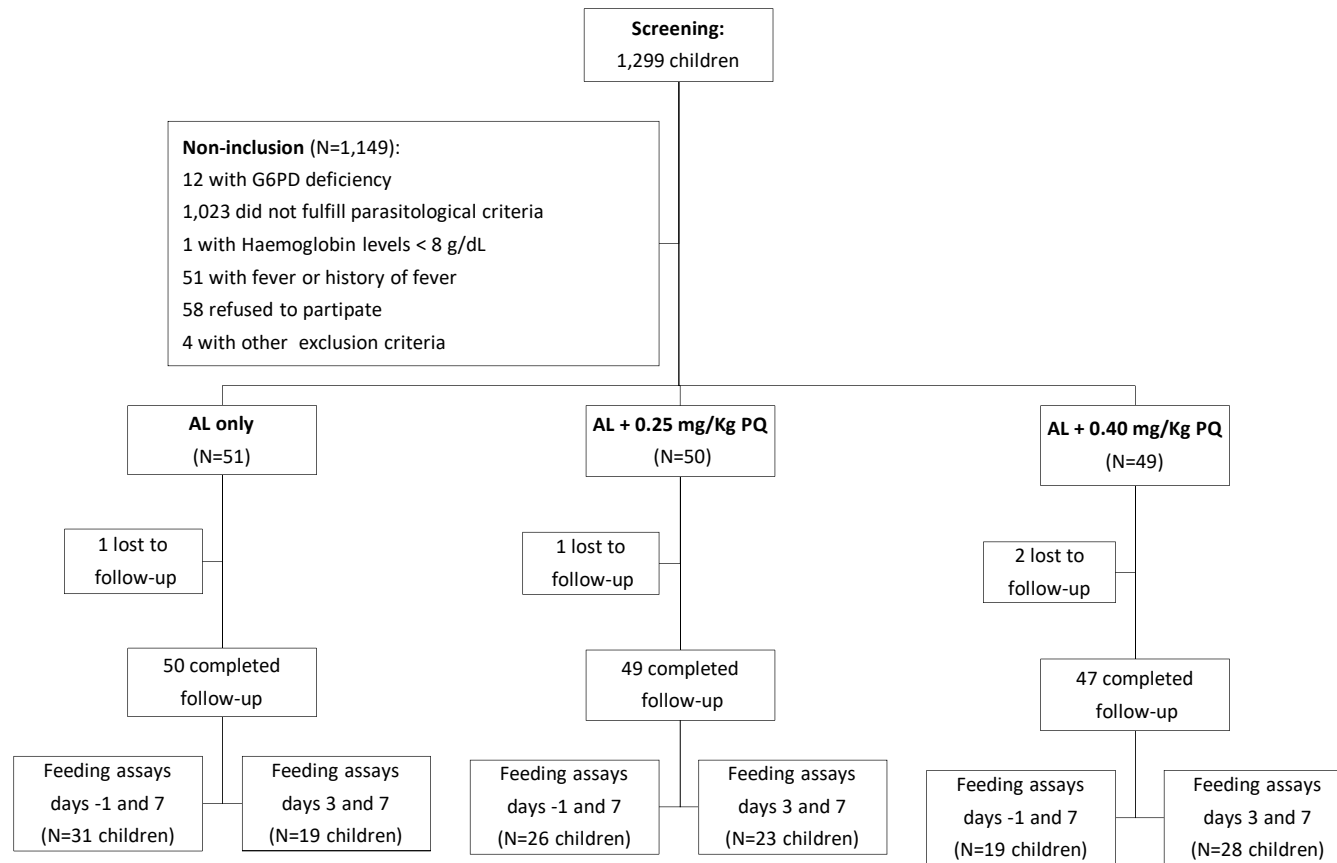


Table 1. Study Population. Means and 95% confidence intervals are presented for haemoglobin levels at enrollment; medians and interquartile ranges are presented for parasite levels (asexual or sexual stages) and age. Study Phase A = presence of asexual parasites (1,000 - 200,000 parasites/ μ l) at screening as an inclusion criterion; Study Phase B = presence of patent gametocytes at screening as an inclusion criterion; AL = artemether-lumefantrine; PQ = primaquine.

	AL only	AL + 0.25 mg/kg PQ	AL + 0.40 mg/kg PQ
<u>Asymptomatic parasite carriers (Study Phase A)</u>			
Female (%)	51.6	52.0	53.4
Age (years)	7.5 (4 – 10)	8 (5 – 10)	8 (4 – 10)
Haemoglobin (g/dL) at enrollment*	10.8 (10.2 – 11.4)	11.3 (10.7 – 11.9)	11.7 (11.2 – 12.2)
Asexual parasite levels at enrollment (parasites/ μ L; Microscopy)	2,972.5 (1,147 – 6,865)	2,503 (939 – 5,798)	3,339 (874 – 7,358)
Gametocyte prevalence (%) at enrollment (Microscopy)	17.7	32.0	20.5
Gametocyte prevalence (%) at enrollment (QT-NASBA)	96.4	92.9	85.5
<u>Asymptomatic gametocyte carriers (Study Phase B)</u>			
Female (%)	43.1	46.0	42.9
Age (years)	8 (5 – 11)	7 (6 – 11)	8 (6 – 11)
Haemoglobin (g/dL) at enrollment*	11.4 (11.1 – 11.8)	11.7 (11.4 – 12.1)	11.5 (11.1 – 11.9)
Asexual parasite levels at enrollment (parasites/ μ L; Microscopy)	488 (226 – 1,670)	957 (248 – 3,004)	908.5 (198 – 2,297)
Gametocyte prevalence (%) at screening (Microscopy)	100	100	100
Gametocyte prevalence (%) at enrollment (Microscopy)	69.4	55.3	83.3
Gametocyte prevalence (%) at enrollment (QT-NASBA)	95.9	93.7	100.0
Gametocyte levels at enrollment (parasites/ μ L)**	17.3 (7.2 – 45.1)	18.6 (7.8 – 75.3)	16.6 (7.2 – 40.0)

* Measured by Hemocue photometer; similar values observed with full blood count assessment using venous samples

** Quantified by qRT-PCR (only children with patent gametocytes on day 0 [N=150] were included [100/150 were enrolled during Study Phase B]; see *Methods*)

Gametocyte prevalence by microscopy was not significantly different between study arms at enrollment, although during Study Phase A it was higher in the 0.25 mg/kg primaquine arm (32.0%) compared to the AL-placebo and the 0.40 mg/kg primaquine arms (17.7 and 20.5%, respectively). Both primaquine arms had lower microscopically-detectable gametocyte prevalences on days 7, 10 and 14, but not on days 2 and 3, compared to the AL-placebo group (**Table 2**). Similar results were obtained when only children with gametocytes on day 0 were included in this comparison (**Additional file 1: Table S1**).

Table 2. Gametocyte carriage during follow-up. Gametocyte prevalences, clearance times and area under the curve (AUC) of gametocyte distributions over time are presented. Gametocyte clearance time: the rate with which malaria sexual stage parasites were cleared was estimated for children with patent gametocytes on day 0; qRT-PCR data were used (N=150; see **Additional file 1**). Gametocyte prevalences were similar in primaquine study arms throughout the follow-up (all P-values > 0.05), except on day 0 (microscopy-based detection; P=0.004) during Study Phase B. AL = artemether-lumefantrine; PQ = primaquine.

Study Phase A (asymptomatic parasite carriers)	AL	AL + 0.25 mg/kg PQ	AL + 0.40 mg/kg PQ	P-values (AL vs.AL+0.25 mg/Kg PQ)	P-values (AL vs.AL+0.40 mg/Kg PQ)
	% (n/N)	% (n/N)	% (n/N)		
Gametocyte prevalence by microscopy					
Day 0	17.7 (11/62)	32.0 (24/75)	20.5 (15/73)	0.08	0.83
Day 2	12.9 (8/62)	16.4 (12/73)	13.7 (10/73)	0.63	1.00
Day 3	9.8 (6/61)	22.9 (16/70)	14.3 (10/70)	0.06	0.59
Day 7	6.7 (4/60)	4.2 (3/72)	0.0 (0/71)	0.70	0.04
Day 10	3.3 (2/60)	1.4 (1/71)	0.0 (0/71)	0.60	0.21
Day 14	0.0 (0/58)	0.0 (0/73)	0.0 (0/71)	-	-
Gametocyte prevalence by Pfis25 QT-NASBA					
Day 0	96.4 (54/56)	92.9 (65/70)	85.5 (53/62)	0.46	0.06
Day 7	46.3 (25/54)	20.3 (14/69)	16.4 (11/67)	0.003	0.001
Study Phase B (patent gametocyte carriers at screening)	AL	AL + 0.25 mg/kg PQ	AL + 0.40 mg/kg PQ	P-values (AL vs.AL+0.25 mg/Kg PQ)	P-values (AL vs.AL+0.40 mg/Kg PQ)
	% (n/N)	% (n/N)	% (n/N)		
Gametocyte prevalence by microscopy					
Day 0	69.4 (34/49)	55.3 (26/47)	83.3 (40/48)	0.21	0.15
Day 2	34.7 (17/49)	20.4 (9/44)	28.9 (13/45)	0.17	0.66
Day 3	28.6 (14/49)	10.9 (5/46)	15.6 (7/45)	0.04	0.15
Day 7	20.4 (10/49)	6.4 (3/47)	6.7 (3/45)	0.07	0.07
Day 10	17.4 (8/46)	4.3 (2/47)	0.0 (0/44)	0.05	0.006
Day 14	13.6 (6/44)	0.0 (0/40)	0.0 (0/38)	0.03	0.03
Gametocyte Prevalence by Pfis25 QT-NASBA					
Day 0	95.9 (47/49)	93.7 (45/48)	100.0 (46/46)	0.68	0.50
Day 7	55.1 (27/49)	20.4 (10/49)	17.0 (8/47)	0.001	<0.001

Table 2 (Continuation)

Only patent gametocyte carriers at enrollment*	AL	AL + 0.25 mg/kg PQ	AL + 0.40 mg/kg PQ	P-values (AL vs.AL+0.25 mg/Kg PQ)	P-values (AL vs.AL+0.40 mg/Kg PQ)
	% (n/N)	% (n/N)	% (n/N)		
Gametocyte Prevalence by Pfs25 qRT-PCR					
Day 0	95.6 (43/45)	98.0 (48/49)	98.0 (49/50)	0.60	0.60
Day 2	93.2 (41/44)	87.5 (42/48)	90.4 (47/52)	0.49	0.72
Day 3	88.4 (38/43)	75.5 (37/49)	92.2 (48/52)	0.18	0.72
Day 7	66.7 (28/42)	34.0 (17/50)	27.8 (15/54)	0.003	<0.001
Day 10	65.8 (27/41)	28.6 (14/49)	20.7 (11/53)	0.001	<0.001
Day 14	42.5 (17/40)	10.2 (5/49)	16.7 (9/54)	0.001	0.01
	Mean (95% CI)	Mean (95% CI)	Mean (95% CI)		
AUC gametocyte densities over time (in gametocytes μL ⁻¹ days)**	14.7 (5.5 – 24.0)	5.5 (1.1 – 10.0)	5.6 (2.1 – 9.1)	-	-
Gametocyte clearance time (in days)	19.7 (14.6 – 24.8)	7.7 (6.3 – 9.1)	8.2 (6.7 – 9.6)	-	-
	AL vs. AL + 0.25 mg/kg PQ	AL vs. AL + 0.40 mg/kg PQ	AL + 0.25 mg/kg PQ vs. AL + 0.40 mg/kg PQ	P-values (AL vs.AL+0.25 mg/Kg PQ)	P-values (AL vs.AL+0.40 mg/Kg PQ)
Difference in gametocyte clearance time (in days)	12.0 (6.7 – 17.3)	11.5 (6.2 – 16.8)	-0.5 (- 2.5 – 1.6)	<0.001	<0.001

* 50 children from Study Phase A and 100 from Study phase B

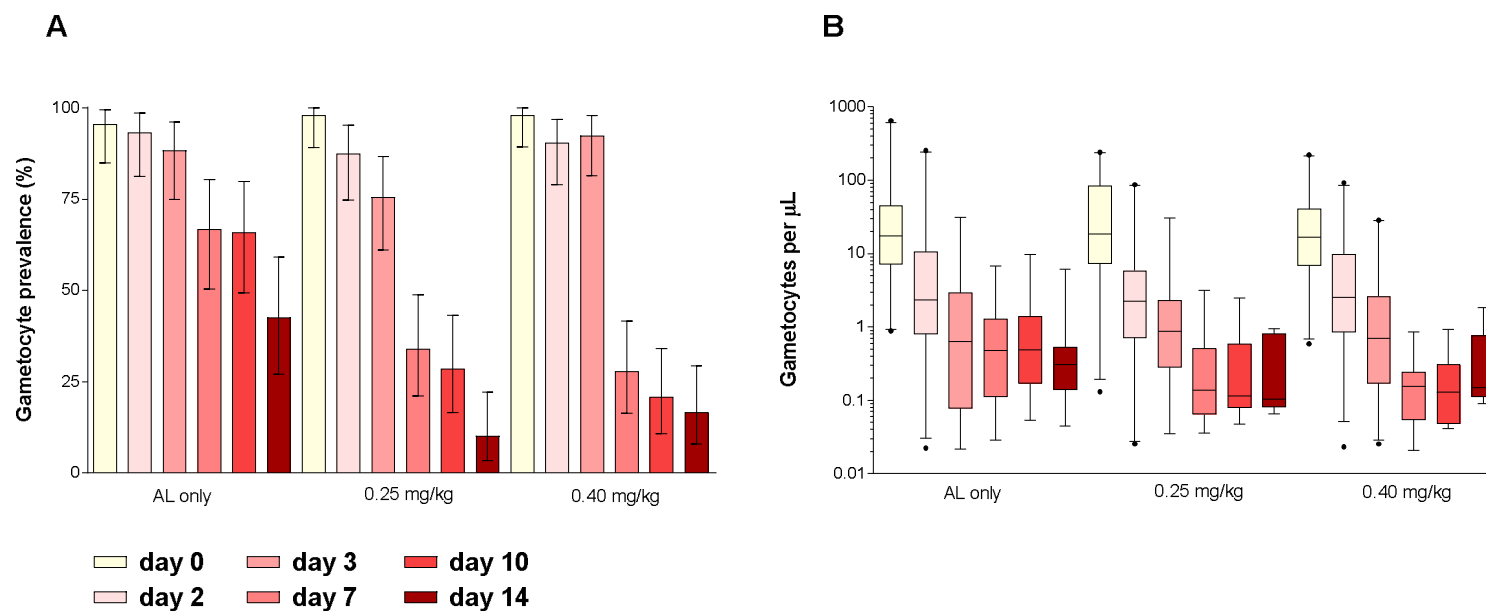
**Only qRT-PCR measurements on days 3, 7, 10 and 14 for children with qRT-PCR data on all these days are included

Molecular detection of gametocytes

Gametocyte prevalences determined by Pfs25 mRNA QT-NASBA were considerably higher than by microscopy (**Table 2**). On day 7, QT-NASBA-determined gametocyte prevalence was 50.5% in the AL-placebo arm, 20.3% in the 0.25 mg/kg primaquine arm ($P < 0.001$, versus control) and 16.7% in the 0.40 mg/kg primaquine arm ($P < 0.001$, versus control; $P = 0.50$, versus 0.25 mg/kg primaquine dose).

Gametocyte prevalences and densities measured by qRT-PCR for children with microscopically detectable gametocytes on day 0 are presented in **Figure 2**. Gametocyte levels in gametocyte-positive visits did not differ between treatment arms on days 0, 2, 3 and 14; on days 7 and 10, the 0.40 mg/kg primaquine arm had lower gametocyte levels compared to the placebo group (**Additional file 1: Table S2**). Gametocyte densities were similar in primaquine arms throughout the follow-up, as gametocyte clearance rates. Children receiving either dosages carried sexual stage parasites for a shorter time compared to the AL only arm (19.7 [14.6 – 24.8], 7.7 [6.3 – 9.1] and 8.2 [6.7 – 9.6] days [mean and 95% CI] to gametocyte clearance for the AL-placebo, the 0.25 mg/kg primaquine and the 0.40 mg/kg primaquine arms, respectively; **Table 2**).

Figure 2. Gametocyte prevalences (A) and densities (B) measured by qRT-PCR in children with patent gametocytes on day 0. 95% confidence intervals are presented in (A). Samples were considered to be gametocyte negative if assigned levels were lower than 0.02 gametocytes per μL . AL = artemether-lumefantrine; 0.25 mg/kg = 0.25 mg/kg primaquine arm; 0.40 mg/kg = 0.40 mg/kg primaquine arm



Infectiousness to mosquitoes

In Study Phase A, when individuals were recruited regardless of gametocytaemia by microscopy, 106 children participated in membrane feeding experiments on days 3 (N= 54), 7 (N= 52), 10 (N=54) and 14 (N=52); during this Study Phase, no mosquitoes developed infection (**Table 3**). In Study Phase B, 149 individuals with patent gametocytes at screening participated in feeding experiments prior to treatment (N=79) and on days 3 (N=70; 19, 23 and 28 in the AL-placebo, the 0.25 mg/kg primaquine and the 0.40 mg/kg primaquine arms, respectively) and 7 (N=144; 49, 49 and 46 in the AL-placebo, the 0.25 mg/kg primaquine and the 0.40 mg/kg primaquine arms, respectively). On average, 45.3 (range 25 - 94) mosquitoes were dissected per assay. 38.0% (30/79) of the experiments conducted prior to treatment resulted in ≥ 1 infected mosquito. In these 30 infectious feeds, the median proportion of infected mosquitoes was 20.0% (IQR 8.0 – 39.0) and median oocyst count per infected mosquito was 5 (IQR 2 – 15). Only one individual infected mosquitoes following treatment, in the AL only arm and infected 4/46 mosquitoes on day 7; this participant, who was also infectious before treatment administration (18/48 infected mosquitoes), had submicroscopic gametocytemia at the time of feeding on day 7.

Table 3. Mosquito feeding assays

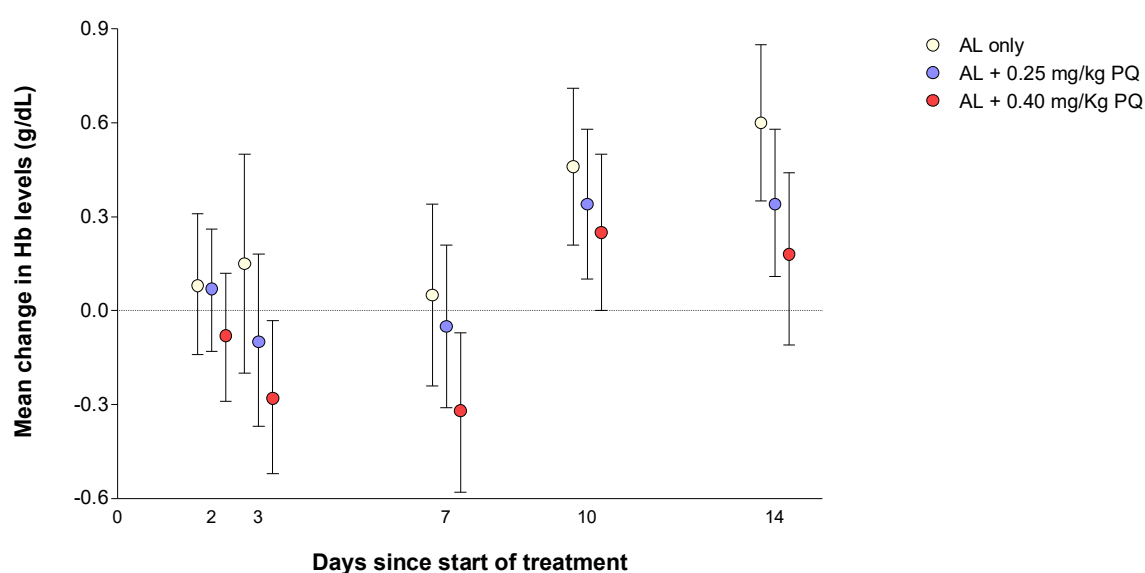
	AL only	AL + 0.25 mg/kg PQ	AL + 0.40 mg/kg PQ
Study phase A			
<i>Number of mosquitoes infected/dissected (proportion)</i>			
Follow-up day			
3	0/755 (0)	0/816 (0)	0/862 (0)
7	0/781 (0)	0/777 (0)	0/767 (0)
10	0/822 (0)	0/848 (0)	0/890 (0)
14	0/855 (0)	0/890 (0)	0/841 (0)
Study phase B			
<i>Number of mosquitoes infected/dissected (proportion)</i>			
Follow-up day			
One day before treatment	207/1402 (0.15)	54/1195 (0.05)	80/897 (0.09)
3	0/863 (0)	0/1053 (0)	0/1281 (0)
7	4/2104 (0.002)	0/2179 (0)	0/2016 (0)

Adverse events and haematological changes in G6PD normal children

57 mild and moderate adverse events were recorded; 8 (7 mild, 1 moderate), occurring in 3, 1 and 4 individuals assigned to the AL-placebo, the 0.25 mg/kg primaquine and the 0.40 mg/kg primaquine arms, respectively, were considered to be possibly related to study participation (**Additional file 1: Table S3**). For 5 children who vomited on days 0 or 1, study drug was re-administered. No serious adverse events were observed.

At enrollment, haemoglobin levels did not differ between treatment arms (**Table 1**). One child had 6 g/dL on day 0, but this was most likely due to measurement error: on day 2, haemoglobin concentration was 10.9 g/dL. Median (IQR) uncorrected reticulocyte percentage at enrollment was 2.0% (1.5 – 2.6). The average maximal fall in haemoglobin levels for children with measurements on all visits (227/360) was larger ($P=0.006$), in absolute value, in the 0.40 mg/kg primaquine arm, -1.21 (95% CI, -1.45 - -0.97, $N=76$) g/dL, compared to the placebo arm, -0.71 (95 % CI, -0.98 - -0.44, $N=72$). Haemoglobin drop for participants receiving the 0.25 mg/kg primaquine dose was -0.96 (95% CI, -1.18 - -0.73, $N=79$; $P=0.16$ and 0.12, versus control and 0.40 mg/kg primaquine arms, respectively). Similar results were obtained when considering maximal percentage drop (**Additional file 1: Table S4**). The lowest haemoglobin values relative to baseline were observed on days 3 and 7 (**Figure 3**). 35 children (9/113 [8.0%], 12/125 [9.6%] and 14/122 [11.5%] in the AL-placebo, the 0.25 mg/kg primaquine and the 0.40 mg/kg primaquine arms, respectively) had haemoglobin drop of 2 or more g/dL (**Additional file 1: Table S5**): in 2, one in each primaquine arm, haemoglobin fell more than 4 g/dL and levels recovered before follow-up was completed. On days 1 and 2, 3 children reported haemoglobinuria. No child had haemoglobin levels < 5 g/dL, and no blood transfusions were required. Renal and liver laboratory abnormalities were detected in 4 and 7 children, respectively (**Additional file 1: Table S6**).

Figure 3. Changes in haemoglobin levels from baseline values. Means and 95% confidence intervals are presented. Haemoglobin levels determined by Hemocue photometer on day 0 were used as baseline measurement; whenever haemoglobin concentrations on day 0 were not available or were only quantified by full blood count analysis on venous samples, Hemocue results at screening were used: the median (IQR) interval between screening and the day 0 of follow-up was 2 (1 – 2) days. AL = artemether-lumefantrine; PQ = primaquine; Hb = haemoglobin.



Discussion

Since 2012, the WHO recommends a single 0.25 mg/kg primaquine dose as gametocytocide but this dosage had never been formally assessed for gametocyte clearance after ACT. In this study, we recruited asymptomatic parasitaemic children, a group that is believed to contribute significantly to transmission in endemic areas [13] and would potentially be given low dose primaquine if used in future community chemotherapy campaigns, and showed that individuals receiving a 0.25 mg/kg primaquine dose in combination with AL had similar gametocyte clearance time compared to children in the 0.40 mg/kg primaquine arm. Both regimens were associated with significantly shorter post-treatment gametocyte circulation compared to the ACT partner drug alone. In the subset of participants who had their infectivity quantified by feeding experiments, infectiousness was effectively reduced after treatment in all arms. Our observations suggest that (i), on the basis of gametocyte carriage, the primaquine dose of 0.25 mg/kg is as effective as the previously evaluated dose, 0.40 mg/kg, and (ii) gametocytemia is not a good predictor of post-treatment infectivity [12]: infectivity is very low after AL and not detected after AL-primaquine.

Previous trials on low dose primaquine's transmission-reducing properties have assessed efficacy based on gametocyte carriage [10]. However, the non-linear relationship between gametocyte levels and mosquito infection rates [22] during experimental infections suggests that reductions in numbers of circulating gametocytes might not be directly translated into changes in transmission potential. Additionally, primaquine might have an impact on infectiousness before sexual stage parasites clearance is observed [12]. Pfs25 mRNA transcripts are more abundantly present in female gametocytes [23, 24] and may also be detected in non-infectious gametocytes [25]. It was recently hypothesized that male gametocytes might be more sensitive to 8-aminoquinolines *in vivo* [26] and, since they represent a smaller fraction of the total gametocyte population compared to female gametocytes [27], their clearance will only minimally influence gametocytaemia by Pfs25 mRNA-based quantification methods but significantly affect infectiousness [28]. Thus, estimating the effect of primaquine on transmission requires direct assessment of infectivity. In this trial, we quantified infectiousness for 255 individuals and 22,894 dissected mosquitoes and adapted our study design to maximize discriminative power by

enriching our study population for individuals with microscopically-detectable gametocytes.

We observed that 38.0% of gametocytaemic individuals infected mosquitoes before treatment. This is within the normal range of infectivity in patent gametocyte carriers [29], although considerably higher infection rates have been reported [22, 30]. After treatment, only one child infected mosquitoes, suggesting that transmissibility was substantially reduced by AL [3]. Low infectivity after AL alone made it impossible to detect an added value of primaquine in this context. The almost complete absence of infected mosquitoes post-treatment contrasts with the limited efficacy of artemisinin derivatives against mature gametocytes, and could be related to lumefantrine, that inhibits male gametocyte exflagellation and reduces oocyst numbers *in vitro* [31]. Factors intrinsic to these infections (e.g., gametocyte sex ratios) or to the host (e.g., transmission-reducing immunity) could also have contributed to this low infectiousness. Another explanation is that membrane feeding experiments, which in general underestimate infectiousness compared to skin feeding assays [29], might not have been sensitive enough to detect very low infectivity. The successful detection of pre-treatment infectiousness, however, suggests that low sensitivity cannot fully explain our findings. Only few studies (summarised in **Additional file 1: Table S7**) assessed the effect of AL on transmission: similar to our observations, in The Gambia, post-treatment gametocyte carriage was observed, but mosquito infections were not detected [3]. In contrast, in Kenya, transmission was observed after AL [6] and clonally complex infections were detected by PCR in infected mosquito guts.

Determining the true impact of primaquine on malaria infectivity should be high on the malaria elimination agenda. Larger clinical studies with appropriate sample sizes to test efficacy outcomes involving infectiousness reduction are necessary. These studies may also determine the optimum timing of primaquine administration. In our study, primaquine was administered on the last day of AL use, to follow the same treatment procedures as a previous dose-finding trial in Uganda, although there are logistical advantages to administering primaquine with the first supervised dose of AL. Ideally, these studies will include symptomatic and asymptomatic malaria infections to demonstrate the efficacy of primaquine in all infections that contribute to the human infectious reservoir for malaria. These studies will be logistically complex

and need to consider all factors that influence infectiousness as they might also influence efficacy estimates: (1) membrane feeding assays underestimate infectivity compared to direct skin feeding assays and consequently low level post-treatment infectivity might be misclassified as non-infectiousness when using this artificial system; (2) the number of mosquitoes used in each assay might not represent the number of mosquito bites an infectious individual would receive in natural settings (especially acknowledging as this is a single time point assessment), and binary outcomes (e.g., “infectious” versus “not-infectious”) might be less relevant than within-individual changes in mosquito infection rates; (3) baseline infectivity will depend on the inclusion criteria and although recruiting individuals with high gametocyte densities would maximize the power to observe changes in infectiousness with treatment, these individuals might not be representative of an “average” infected person. As duration and intensity of infectiousness are likely to depend on host age and transmission setting, individuals from different ages and study sites, representing a wide spectrum of endemicities, would need to be recruited.

Primaquine-induced haemolysis in individuals with G6PD deficiency is a concern for control programs and one major reason why this drug is not widely used in Africa. Here, a rapid diagnostic test was used to exclude individuals with this condition. No cases of severe haemolysis were observed, corroborating previous observations made in symptomatic children with normal G6PD activity receiving a single 0.40 mg/kg primaquine dose [10]. However, contrary to this previous trial undertaken in Uganda, children receiving 0.40 mg/kg primaquine in this study had larger haemoglobin drops compared to the placebo group, suggesting that mild haemolysis might have occurred. While our study provides detailed haematological assessment after primaquine administration in G6PD normal non-anaemic children with asymptomatic falciparum infections, G6PD deficiency could lead to greater haemoglobin drops and studies recruiting G6PD deficient individuals are necessary.

Conclusion

The ultimate goal of using primaquine in falciparum malaria in conjunction with ACT is to minimise post-treatment malaria transmission, reduce the transmission of artemisinin-resistant parasites and accelerate attempts to eliminate malaria [32].

Although mathematical models have been developed to estimate the potential impact of this drug on population-level transmission [33], its effect on infectiousness needs to be quantified. In this study, children receiving 0.40 mg/kg or 0.25 mg/kg primaquine doses cleared gametocytes with similar rates; both treatment regimens reduced gametocyte carriage compared to ACT alone. Feeding assays, however, indicate that infectiousness is considerably reduced by AL alone, suggesting that the benefit of adding primaquine to first-line antimalarials is influenced by partner drug.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions:

TB and CD designed the study. BPG, ABT, AO, WMG, IN, DS, ACE, AD, ECB, SBS, CD and TB were involved in data acquisition. KL performed molecular assays to detect and quantify gametocytes. BPG, JB, HP and TB contributed to statistical analysis. BPG, ABT, CD and TB wrote the first draft of this manuscript. All authors reviewed and approved the final version of this manuscript.

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Supplementary Appendix

Single Low Dose Primaquine to Reduce Gametocyte Carriage and *Plasmodium falciparum* Transmission after Artemether-Lumefantrine in Children with Asymptomatic Infection: A Randomised, Double-Blind, Placebo-Controlled Trial

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Non-linear model to estimate gametocyte clearance time

A non-linear model was fit in SAS 9.3 (SAS Institute, North Carolina, United States) to estimate the rate of gametocyte clearance in study participants allocated to different treatment regimens:

$$G_t = e^{-rt} \quad [1]$$

where G_t represents the proportion of individuals with gametocytes at time t . r is the rate with which gametocytes are cleared and depends on the treatment received:

$$r = e^{(\ln r_0 + aI_1 + bI_2)} \quad [2]$$

where r_0 corresponds to the clearance rate in the artemether-lumefantrine only group; and I_1 and I_2 are indicator variables representing the 0.25 mg/kg and the 0.40 mg/kg primaquine dose arms, respectively. a and b represent the effects of the different primaquine doses on the rate of gametocyte clearance and are estimated from the data.

Haematological changes after treatment

Finger-prick measurements

219 children had haemoglobin levels quantified by Hemocue photometer on day 0. For those study subjects without Hemocue-derived haemoglobin levels in the beginning of the follow-up, measurements done at screening were used as baseline: the median (interquartile range) interval between screening and the day 0 of follow-up was 2 (1 – 2) days. The majority (227/360) of study participants had haemoglobin levels measured on five follow-up visits after day 0; 9 children had 2 or less measurements. When all enrolled individuals are considered, regardless of the number of Hemocue measurements during follow-up, the average (95% CI) maximal fall in haemoglobin concentrations after treatment initiation was -0.60 (-0.81 - -0.39), -0.75 (-0.95 - -0.56) and -0.84 (-1.08 - -0.61) g/dL in the AL-placebo arm, the 0.25 mg/kg primaquine dose arm and the 0.40 mg/kg primaquine dose arm, respectively (N=355, P=0.12).

Venous blood measurements

During follow-up, haematological parameters were also measured on venous blood samples collected on days 0, 3 and 7. When using haemoglobin levels measured on these venous samples to assess haemolysis, the average (95% CI) maximal fall in

haemoglobin concentrations after treatment initiation was -0.19 (-0.30 - -0.07), -0.30 (-0.45 - -0.14) and -0.33 (-0.45 - -0.20) g/dL in the AL-placebo arm (N=110), the 0.25 mg/kg primaquine dose arm (N=122) and the 0.40 mg/kg primaquine dose arm (N=119), respectively (P=0.32).

Reticulocytes

16 children had reticulocyte levels higher than 5% at enrolment. During follow-up, in 47 scheduled visits (17 on day 3 and 30 on day 7), reticulocyte levels were above 5%: 14, 18 and 15 in the AL-placebo, 0.25 mg/kg primaquine and 0.40 mg/kg primaquine arms, respectively. Median absolute reticulocyte count at enrolment was 88 (range 66 – 113) $\times 10^3$ reticulocytes per μL ; absolute levels increased on day 7 versus day 0 in 78/101, 93/116 and 89/110 children in the AL-placebo, 0.25 mg/kg primaquine and 0.40 mg/kg primaquine arms, respectively. There was a positive correlation between increase in reticulocyte levels during the first week of follow-up and haemoglobin drop by day 7 in the primaquine arms (Spearman's rank correlation coefficient 0.18 [P=0.03], N=141).

Parasitological criterion

20 children with less than 1,000 asexual falciparum parasites per μL and no gametocytes at screening were mistakenly enrolled. They were equally distributed in the different study arms: 6, 7 and 7 were enrolled in the AL-placebo, the 0.25 mg/kg primaquine and the 0.40 mg/kg primaquine arms, respectively. 3, one from each study arm, of these 20 children had gametocytes on day 0 and were included in the gametocyte clearance analysis. Additionally, one child, from the AL-placebo group, had *P. malariae* infection detected by microscopy at enrolment, but not during follow-up visits. Data from these children were included in analyses presented in this manuscript.

Tables

Table S1. Prevalence of microscopically-detectable gametocytes during follow-up in children with patent gametocytes on day 0. AL = artemether-lumefantrine; PQ = primaquine.

	AL % (n/N)	AL + 0.25 mg/kg PQ % (n/N)	AL + 0.40 mg/kg PQ % (n/N)
<i>Day 2</i>	38.6 (17/44)	35.4 (17/48)	36.5 (19/52)
<i>Day 3</i>	34.1 (15/44)	35.4 (17/48)	23.5 (12/51)
<i>Day 7</i>	20.9 (9/43)	10.4 (5/48)	5.8 (3/52)
<i>Day 10</i>	19.5 (8/41)	4.1 (2/49)	0.0 (0/51)
<i>Day 14</i>	15.4 (6/39)	0.0 (0/44)	0.0 (0/45)

Table S2. Gametocyte levels (per μL) measured by qRT-PCR during gametocyte-positive visits in children with patent gametocytes on day 0. AL = artemether-lumefantrine; PQ = primaquine.

	AL Median (IQR, N)	AL + 0.25 mg/kg PQ Median (IQR, N)	AL + 0.40 mg/kg PQ Median (IQR, N)	AL vs. AL + 0.25mg/kg PQ P-value	AL vs. AL + 0.40 mg/kg PQ P-value
<i>Day 0</i>	17.26 (7.21 – 45.08, 43)	18.62 (7.76 – 75.27, 48)	16.63 (7.18 – 39.99, 49)	0.58	0.83
<i>Day 2</i>	2.33 (0.81 – 10.21, 41)	2.25 (0.75 – 5.83, 42)	2.52 (0.85 – 9.70, 47)	0.57	0.97
<i>Day 3</i>	0.64 (0.08 – 2.64, 38)	0.87 (0.30 – 2.28, 37)	0.70 (0.17 – 2.49, 48)	0.35	0.67
<i>Day 7</i>	0.48 (0.12 – 1.28, 28)	0.14 (0.08 – 0.45, 17)	0.15 (0.05 – 0.24, 15)	0.12	0.02
<i>Day 10</i>	0.49 (0.17 – 1.40, 27)	0.12 (0.08 – 0.54, 14)	0.13 (0.05 – 0.31, 11)	0.07	0.008
<i>Day 14</i>	0.30 (0.18 – 0.50, 17)	0.10 (0.10 – 0.66, 5)	0.15 (0.12 – 0.53, 9)	0.56	0.77

Table S3. Adverse Events by treatment arm. In (A), adverse events (AEs) are presented by severity and causality; in (B), by clinical condition. AL = artemether-lumefantrine; PQ = primaquine.

(A)

Adverse Events by Severity and causality*	AL only	AL + 0.25 mg/kg PQ	AL + 0.40 mg/kg PQ
Any AE	15	18	24
Mild AEs	7	7	9
Mild AEs possibly related to study	2	1	4
Moderate AES	8	11	15
Moderate AEs possibly related to study	1	0	0
Severe AEs	0	0	0

*In total 48 children developed AEs during follow-up; 8 had 2 or more AEs.

(B)

Adverse Event	AL only	AL +	AL +
		0.25 mg/kg PQ	0.40 mg/kg PQ
Bronchitis	2	4	6
Fever	2	4	5
Abdominal Pain	1	1	0
Vomiting ^a	2	2	2
Uncomplicated Malaria	2	1	2
Rhinitis/ Rhino-Bronchitis	1	1	1
Otitis	0	0	2
Dysentery	1	2	3
Epistaxis	0	0	1
Dental Pain	1	0	0
High transaminase levels	1	0	0
Palpebral inflammation	1	0	0
Muscle pain (Neck)	0	1	0
Wound	1	1	1
Trauma on left foot	0	1	0
Inflammation (left foot)	0	0	1

^a 5/6 vomiting episodes occurred on days 0 or 1; for all these 5 visits, study drug was re-administered. 1 child vomited on day 3 after treatment initiation

Table S4. Maximal percentage drop in haemoglobin levels, relative to baseline (enrolment or screening), by treatment arm. AL = artemether-lumefantrine; PQ = primaquine.

Maximal % drop in haemoglobin	
Mean % (95% Confidence Interval)	
<i>AL only</i>	5.7 (3.3 - 8.1)
<i>AL + 0.25 mg/kg PQ</i>	7.8 (5.8 - 9.8)
<i>AL + 0.40 mg/kg PQ</i>	9.9 (7.9 - 11.9)

Table S5. Number of participants with substantial haemoglobin drops (2 or more g/dL) during follow-up (N=35). AL = artemether-lumefantrine; PQ = primaquine.

	Haemoglobin drop (in g/dL)		
	2 - 3	3 - 4	> 4
<i>AL only</i>	6	3	0
<i>AL + 0.25 mg/kg PQ</i>	11	0	1
<i>AL + 0.40 mg/kg PQ</i>	9	4	1

Table S6. Laboratory abnormalities during follow-up (days 3 and 7). In addition to the findings presented in this table, 7 children presented white blood cell counts below 4,000 cells per μL during follow-up: 2, 3 and 2 in the AL-placebo arm, the 0.25 mg/kg primaquine dose arm and the 0.40 mg/kg primaquine dose arm, respectively. AL = artemether-lumefantrine; PQ = primaquine.

White blood cell counts > 15,000 / μL	
Treatment arm	Number of children
<i>AL only</i>	3
<i>AL + 0.25 mg/kg</i>	2
<i>AL + 0.40 mg/Kg</i>	1
Platelet count < 150,000 / μL	
Treatment arm	Number of children
<i>AL only</i>	4
<i>AL + 0.25 mg/kg</i>	5
<i>AL + 0.40 mg/Kg</i>	4
Creatinine levels above upper limit of normal range ^{*,ϕ}	
Treatment arm	Number of children
<i>AL only</i>	2
<i>AL + 0.25 mg/kg</i>	1
<i>AL + 0.40 mg/Kg</i>	1
Liver transaminases levels above upper limit of normal range ^{‡,\square}	
Treatment arm	Number of children
<i>AL only</i>	4
<i>AL + 0.25 mg/kg</i>	2
<i>AL + 0.40 mg/Kg</i>	1
Total bilirubin levels above 20 $\mu\text{mol/L}$	
Treatment arm	Number of children ^a
<i>AL only</i>	2
<i>AL + 0.25 mg/kg</i>	5
<i>AL + 0.40 mg/Kg</i>	7

* < 6 years: 44.2 $\mu\text{mol/L}$; 6 - 10 years: 53.0 $\mu\text{mol/L}$; 10-15 years: 61.9 $\mu\text{mol/L}$

ϕ 2/4 had normal creatinine levels by the end of the follow-up

[‡]AST: < 6 years: 79 U/L; 6 - 10 years: 74 U/L; 10-15 years: 69 U/L

ALT: < 6 years: 80 U/L; 6 - 10 years: 78 U/L; 10-15 years: 76 U/L

^{\square} In 6/7 children with liver transaminases above normal range, levels decreased during the follow-up

^a 3 children, one in each treatment arm, had bilirubin levels above 20 $\mu\text{mol/L}$ at enrolment

Table S7. Summary of previous studies assessing post-artemether lumefantrine transmission potential. Mosquito infection rates in the study by Ouédraogo and colleagues were based on PCR; the other studies listed in the table used microscopy to assess oocyst positivity.

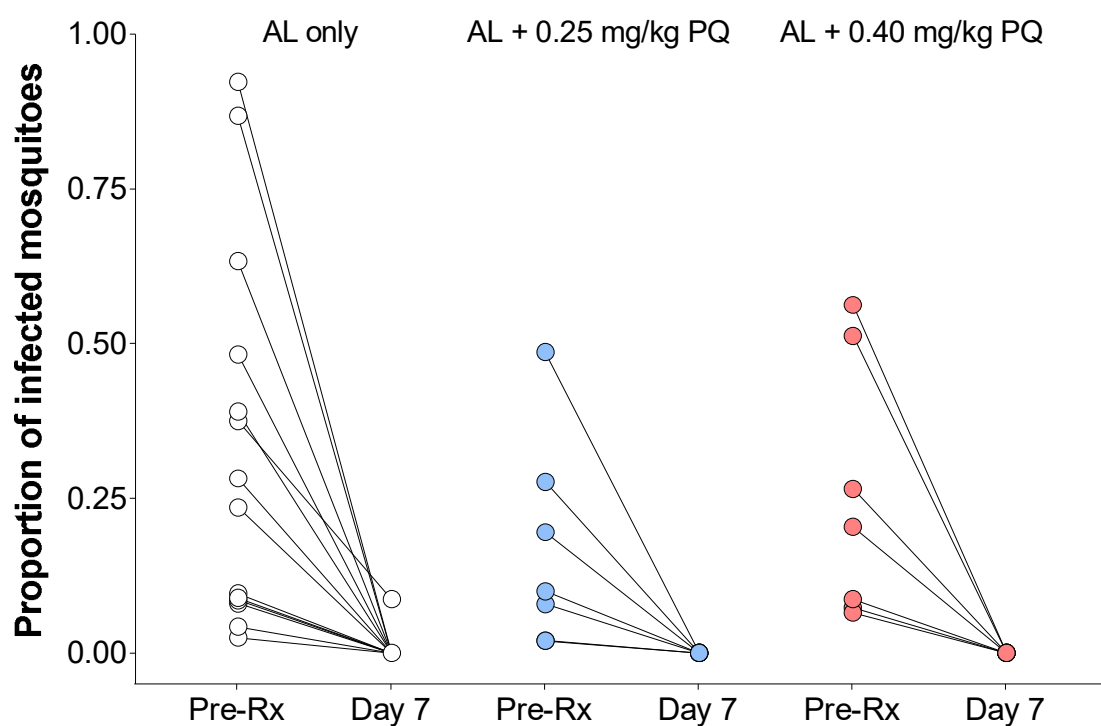
	Study type	Setting	Inclusion trial	Gametocyte positivity required for feeding assay?	Number of assays	Time from treatment initiation (feeding assay)	Number of infected mosquitoes in feeding assay (AL only group)	Number of infectious individuals in feeding assay (AL only group)
<i>Sutherland et al. 2005</i>	RCT*	Gambia	Children with uncomplicated malaria	Yes	10	Day 7	0/196 (0)	0/10
<i>Bousema et al. 2006</i>	RCT	Kenya	Children with uncomplicated malaria	No	25	Day 14	27/750 (3.6%)	15/25
<i>Sawa et al. 2013</i>	RCT	Kenya	Children with uncomplicated malaria	No	77	Day 7	43/2293 (1.9%)	24/77
<i>Ouédraogo et al. 2015</i>	RCT	Burkina Faso	Asymptomatic adults	No**	68	Days 1 and 7	4/560 (0.7%)	-

* randomised controlled trial

** Although gametocyte positivity was not a criterion for inclusion in feeding assays, only children with positive Pfs25 mRNA NASBA results had mosquito infection status assessed
 Sutherland CJ et al. Reduction of malaria transmission to Anopheles mosquitoes with a six-dose regimen of co-artemether. PLoS Med 2005, 2(4):e92
 Bousema JT et al: Moderate effect of artemisinin-based combination therapy on transmission of Plasmodium falciparum. J Infect Dis 2006, 193(8):1151-1159
 Sawa P et al. Malaria transmission after artemether-lumefantrine and dihydroartemisinin-piperaquine: a randomized trial. J Infect Dis 2013, 207(11):1637-1645
 Ouédraogo et al. Efficacy and safety of the mosquitocidal drug ivermectin to prevent malaria transmission after treatment: a double-blind, randomized, clinical trial. Clin Infect Dis. 2015.

Additional Figure

Additional Figure 1. Near absence of infectiousness post-treatment. The y-axis corresponds to the proportion of mosquitoes infected in membrane feeding assays. Only data from individuals who infected at least one mosquito before treatment administration ('Pre-Rx') are included. No study participants, except one from the AL only arm, were infectious on day 7 after enrolment.



Chapter 6, Discussion

In this chapter, I summarise the main findings presented in this document and consider aspects of the studies described in **Chapters 3 – 5** that were not discussed in detail in chapter-specific discussions.

Infectiousness of human populations to mosquitoes in malaria endemic areas

In the epidemiological theory of mosquito-borne infections [1], two human infection-related parameters are thought to directly influence transmission or more precisely the basic reproduction number: the probability that a mosquito bite on an infected individual results in mosquito infection and the duration of the infectious period. The infectiousness surveys performed in Burkina Faso and Kenya (**Chapter 3**) generated data that allow estimation of the first parameter during dry and rainy seasons. Indeed, for those studies, individuals were recruited regardless of parasite status by standard or molecular diagnostics and participated in membrane feeding experiments that quantify malaria infectiousness to mosquitoes. In Burkina Faso, the prevalences of infectiousness (4.9 and 6.4% during dry and wet seasons respectively) were considerably lower compared to the prevalences of malaria infection by molecular assays (50.5 and 83.8%); and only 0.9 and 1.4% of mosquitoes feeding on parasite-positive individuals during the dry and wet seasons, respectively, were estimated to acquire infection after adjustment for demographics. In the Kenyan study sites, a similar pattern was observed: while one third of the study population in Kilifi and ~25% of study participants in Mbita had falciparum parasites detected by *I8S* qPCR and microscopy, respectively, the prevalences of infectivity in both study sites were below 5%.

The reason why infectiousness to mosquitoes is infrequent, even in areas with high transmission, is not well understood. The capacity of falciparum parasites to produce gametocytes varies in different clinical isolates [2], however, in theory, asexual stage parasites of all circulating strains should be able to develop into gametocytes otherwise these strains would get extinct. Gametocyte prevalences by *Pfs25* mRNA QT-NASBA confirm that most infected individuals in these surveys carried sexual stage parasites,

although the skewed distribution of gametocyte densities (**Chapter 3, Additional Figure 1**) indicates that in the vast majority of these infections there were too few circulating gametocytes to infect mosquitoes at the moment of sampling. An important question is whether these ‘snapshots’[3] of gametocyte densities at the time of sampling accurately represent the average densities of gametocytes during individual infections or during a specified period of time – in other words, how variable are gametocyte densities over time in natural infections? Malariotherapy data [4] indicate that gametocyte patency often occurs during or immediately after peaks of asexual stage parasitemia and is rarely detectable by microscopy between consecutive asexual parasitaemia waves. Short-term systematic, rather than random, variation related to hypothesised circadian patterns of gametocyte circulation [5] or infectivity [6] could also have influenced infectiousness measurements as feeding assays were performed only during the day. As mentioned in **Chapter 1**, there is limited evidence for this from studies with intensive sampling over a period of a few days. Possible mechanisms for such variation could involve: 1) circadian relocation of gametocytes in different vascular beds; 2) circadian variation in release of newly mature gametocytes from bone marrow into circulation.

Another possible explanation for the relatively low prevalences of infectiousness observed in the xenodiagnostic surveys described in **Chapter 3** and in previous xenodiagnostic surveys (reviewed in [7]) is that experimental mosquito infections might not be sensitive enough to detect very low infection probabilities. Studies that concomitantly performed membrane feeding experiments and skin feeding assays, where mosquitoes feed on the skin of infected volunteers, indicate that membrane-based assays are less sensitive than skin feeding experiments [8] and might underestimate infectiousness. However, xenodiagnostic studies that used skin feeding found prevalences of infectiousness that are similar to those estimated by studies using membrane feeding [9-12]. The number of mosquitoes dissected in feeding assays is another variable that influences the interpretation of infectiousness assessments, particularly when these are expressed as prevalence of infectivity (capacity to infect at least one mosquito). The number of mosquitoes dissected in each assay should reflect actual exposure over a specified period of time in natural settings. The transmission studies in Burkina Faso used relatively high numbers of mosquitoes (median [IQR] number of dissected mosquitoes per experiment during dry and wet

season surveys, 91 [79 – 97] and 41 [35 – 46], respectively) compared to other xenodiagnostic studies [12]. Of note, field estimates from the same area (**Chapter 4**) indicate that these numbers of dissected mosquitoes correspond to the numbers of mosquito bites individuals would receive over a period of three months and one month, respectively. A more robust metric compared to the categorisation of individuals as infectious or non-infectious and that should be reported in xenodiagnostic studies is the proportion of mosquitoes infected in individual assays. However even this measure is influenced by the number of mosquitoes in each feeding experiment, which determines the lower limit of infectivity quantification. All these limitations need to be considered when interpreting xenodiagnostic studies of malaria and other vector-borne pathogens [13].

In summary, the distribution of gametocyte densities during infections is a likely explanation of why, at the time of surveys, only a few individuals infect mosquitoes. However, infectivity assessments should be interpreted with caution since gametocyte densities change over time and individuals who were not infectious on a particular day might become infectious on the following day (and vice-versa), and over one week, for example, a much higher proportion of the population might be infectious at least one night. Studies quantifying fluctuations in infectivity over different periods of time would allow accurate interpretation of cross-sectional infectivity assessments, and estimation of a cumulative measure of contribution to transmission.

Infectiousness of specific populations

Pregnancy malaria

The studies presented in **Chapter 3** did not specifically quantify malaria infectiousness during pregnancy. Pregnant women, however, are a likely source of malaria transmission due to their high parasite densities as well as their attractiveness to *Anopheles* mosquitoes. In an opinion manuscript [14] to which I contributed (the manuscript is presented in *Appendix*), these and other factors influencing malaria transmission potential during pregnancy, such as sulfadoxine-pyrimethamine (SP) resistance in parasite populations, which reduces parasitological efficacy of intermittent presumptive treatment with SP, are described and arguments, put forward for the inclusion of pregnant women in future mass treatment campaigns. Indeed,

evidence from recent clinical trials [15] that artemisinin-based combination therapies (ACTs) can be safely used to treat malaria infections during pregnancy indicates that treatment of infected pregnant women identified by community-wide screening of placental infections with rapid diagnostic tests, which are sensitive to detect most infections during pregnancy, could improve the likelihood of success of mass drug administration campaigns aiming to interrupt transmission and also prevent pregnancy malaria-associated morbidity [14].

Clinical malaria

Whether individuals with malaria symptoms are, as a group, a non-negligible source of infection to mosquitoes is an important question for malaria control programmes, that need to make decisions on how to best allocate their resources to have a maximum impact on transmission levels and morbidity. A limitation of the xenodiagnostic surveys described in **Chapter 3** is that the overwhelming majority of malaria-infected study participants were asymptomatic, which prevented the estimation of the infectiousness of individuals with clinical malaria. While in vivax infections gametocytes have a short maturation period and a high proportion of individuals with clinical disease are infectious when they present to healthcare facilities [16], in falciparum malaria the long maturation of gametocytes suggests that symptomatic individuals might not necessarily be infectious if they seek treatment early during their infection. In fact, the presence of mature gametocytes in the peripheral blood at the moment of health seeking behaviour, which depends among other things on the duration of the sexual maturation period and how fast the symptoms-causing asexual parasite density increases, was highly predictive of infectiousness in a hospital-based study undertaken in Cambodia [17]. Interestingly, in Kenya, gametocytaemic children with symptoms were less likely to infect mosquitoes compared to asymptomatic gametocytaemic children, suggesting that factors linked to the development of symptoms, such as parasite virulence factors or host responses associated with immunopathology, might affect gametocyte fertility. In addition to differences in the duration of the infectious period and in the ‘per gametocyte’ infectivity, in some areas symptomatic and asymptomatic malaria cases might also be associated with distinct epidemiological processes: in Zambia, where transmission is approaching elimination levels, parasites infecting asymptomatic individuals were genetically different from those infecting individuals with symptoms [18], which indicates that in some areas

there might exist parallel circulation of endemic strains, which would presumably be associated with asymptomatic infections, and strains that often cause symptoms (e.g. imported parasites against which no strain-specific immunity exists in the community). In January 2016, I contributed to a letter to the *Journal of Infectious Diseases* on the importance of malaria transmission from clinical cases [19] (letter included in the *Appendix*).

Heterogeneity in mosquito exposure and spread of parasites at the community level

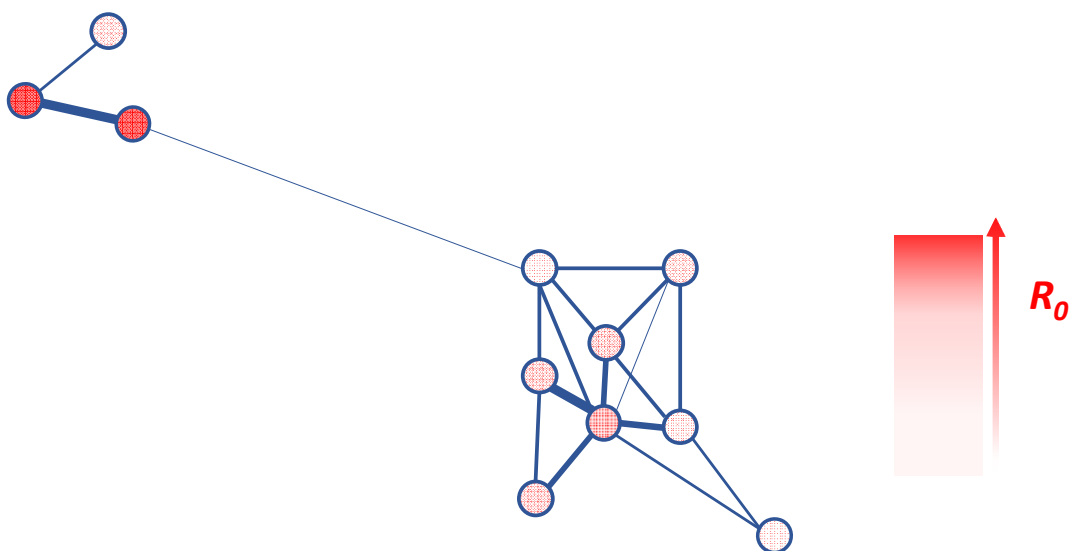
In **Chapter 4**, data on exposure to *Anopheles* mosquitoes were presented. Briefly, bloodfed mosquitoes were collected from households in an area with seasonal malaria transmission and the sources of mosquito blood meals were identified by DNA fingerprinting. In the study area, heterogeneity in exposure was observed at two different levels: the number of bloodfed mosquitoes collected in each study house varied considerably, and individuals living in the same house were often linked to very different numbers of mosquito blood meals. Corroborating findings from previous studies that used other methods of mosquito exposure assessment (e.g. direct observation or ABO blood group typing), the entomological study in Burkina Faso showed that adults are more often exposed to *Anopheles* mosquitoes than children. Age-specific estimates of exposure also allowed for a better quantification of the contributions of different age groups to local malaria transmission (**Chapter 3**). Indeed, after accounting for infectiousness, demographics and vector exposure, schoolchildren were estimated to contribute 41.1 – 45.8% of transmission in our study sites in Burkina Faso, and adults, 51.3 – 55.5%. These results are informative for control interventions: for example, by extending the age limit for administration of seasonal malaria chemoprevention to include schoolchildren, in addition to reductions in morbidity, decreases in community-wide transmission might also occur.

As a consequence of both between- and within-household variation in exposure, a high proportion of the study population was not linked to any mosquito blood meals, especially at the end of the wet season. Our data suggests that there are two mechanisms that could explain this observation: all individuals living in households where vector density is low have limited indoor exposure to mosquitoes; and in houses with high mosquito counts a subset of residents might remain unexposed. Conversely,

some individuals in Balonghin received considerably more mosquito bites than the rest of the population. These individuals are likely to be important sources of infection to other members of their households and locally: by being frequently sampled by different mosquitoes, rather than receiving repeated bites from the same mosquitoes, which would only occur where mixing of malaria vectors is limited (for a clear explanation of the differences between the concepts of heterogeneity and mixing see [20]), these highly-exposed individuals would 1) be more often infected, 2) as a result be more often infectious, and 3) due to their level of exposure have more opportunities to transmit malaria parasites to previously unencountered (uninfected) mosquitoes .

One aspect of the transmission of parasites in a community that was not captured by the mosquito collection studies in Burkina Faso is parasite movement: while spatial variation in vector density and host factors influencing exposure to mosquitoes, such as bed net use, might result in areas where parasite fitness is high (equivalent to $R_0 > 1$), migration of parasites from these areas might sustain or boost transmission in other, less receptive, areas [21]. Although at a large scale (i.e. countries) human movement is more likely to drive parasite introductions, at a village scale, mosquito movement is, presumably, highly relevant [22]. If areas with high mosquito density and high infectiousness prevalence only rarely export infections to the rest of the community, due to distance or factors influencing mosquito movement, households with lower exposure to mosquitoes or with fewer infectious individuals but higher connectivity might be more important in sustaining transmission locally (**Box 1**). Quantifying mosquito movement at different scales would inform the design of targeted interventions to reduce malaria transmission as it would allow the estimation of distance ranges within which mosquitoes acquiring infection in particular households are likely to cause secondary human infections. These distance ranges might depend on mosquito movements, relative locations of oviposition sites and human dwellings, and household-level vector densities.

Box 1. Malaria transmission in networks of households. Circles represent houses in a hypothetical village with endemic malaria transmission, and the lengths of the blue lines are proportional to distances between houses. Each circle's colour represents the household-level malaria transmission potential and is dependent on local vector density, household characteristics and human behaviour that influence exposure to *Anopheles* mosquitoes and prevalence of infectiousness. The widths of the lines connecting different houses depend on the flux of *Anopheles* mosquitoes from one house to another. In this hypothetical setting, the houses with highest transmission potential (top left), despite being an important source of infected mosquitoes locally, are not major contributors to transmission that occurs in the other cluster of households, due to the limited connectivity (in terms of mosquito movement) between those houses and the rest of the village. Indeed, a household with lower transmission potential but with higher flux of mosquitoes leaving to the rest of the village could be more important to the maintenance of transmission in the total area.



Possible interactions between mosquito exposure, human immunity and infectivity

Transmission opportunities and capacity to infect mosquitoes do not necessarily coincide: while children are on average more infectious than adults, adults are more often exposed to mosquitoes. Since the two Burkina Faso studies described in **Chapters 3** and **4** did not recruit the same individuals, we cannot exclude however that within age groups infectious individuals were those who received more mosquito bites – to become infectious, an individual would have received at least one infective bite few weeks or months earlier. On the other hand, individuals living in areas or houses with persistently high vector densities might become infected more often and develop immunity that controls parasitemia faster compared to individuals living in areas with lower vector densities. An association between long term exposure to malaria vectors and immunity development could have two opposite consequences: falciparum parasites and gametocytes densities might be lower in infected individuals living in high-exposure areas compared to age-matched individuals in areas with less frequent *Anopheles* exposure; and anti-disease immunity [23] (i.e. immunity that prevents symptoms after blood stage infection establishment) might also develop faster in areas with higher exposure to mosquitoes, which could influence infection, and infectiousness, duration by postponing treatment seeking behaviour. Studies that longitudinally assess household-level and individual-level mosquito exposure and how these relate to gametocyte levels in repeated infections and to the risk of symptom development during blood stage infections will help to understand the long-term impact of heterogeneity in mosquito exposure on other transmission related phenotypes.

Primaquine and residual malaria transmission

Chapter 5 described an efficacy clinical trial that assessed the effect of low dose primaquine on both gametocyte clearance, determined by RNA-based molecular assays, and infectivity, quantified in membrane feeding assays. Children receiving a single primaquine dose (0.25 mg/kg or 0.40 mg/kg) cleared gametocytes faster than children who received artemether-lumefantrine alone. Membrane feeding assays performed in the same study, however, suggest that artemether-lumefantrine alone can block transmission efficiently (**Chapter 5 Additional Figure 1**), which could be

linked to lumefantrine's effect on exflagellation [24]. The transmission blocking activity of artemether-lumefantrine contrasts with the more limited effect of dihydroartemisinin-piperaquine on malaria transmission: in Mali, highly gametocytaemic individuals who received dihydroartemisinin-piperaquine were often infectious after treatment initiation, unless primaquine was co-administered [25]. The faster clearance of gametocytes, the added value in blocking transmission when used in combination with a drug that has limited activity against transmission stages as well as the growing evidence that the WHO-recommended dose is safe, all this indicates that primaquine could be used by malaria elimination programmes to accelerate reductions in transmission levels.

Community-wide chemotherapy approaches that involve treating individuals based on (mass screening and treatment, MSAT) or regardless of (mass drug administration, MDA) their parasite statuses are being considered and used by malaria elimination programmes. In these mass treatment campaigns, several thousand individuals receive treatment simultaneously and some remain or become infectious post-treatment, with the frequency of residual infectiousness depending on the drug being used [26]. The objective of these campaigns is to eliminate malaria in a particular region by clearing all parasites in humans to prevent new mosquito infections; even a small number of individuals carrying transmissible gametocytes for a few days after drug administration could lead to continuity of transmission. By co-administering primaquine, the likelihood of persistence of parasites at a community level would be minimised. The data presented in **Chapter 4** suggest that some individuals have more transmission opportunities, presumably also after antimalarial administration, and demographically and spatially targeting primaquine administration could be considered where there are safety concerns linked to G6PD deficiency: for example, primaquine could be used as part of MDA campaigns where vector density is high but not necessarily in areas associated with lower vector exposure.

Conclusion

The studies presented in this document, results of efforts of dozens of scientists, aim to provide a comprehensive description of malaria infectiousness at a population level and of heterogeneity of exposure to *Anopheles* mosquitoes. The main findings are: (1)

infectivity is not common in randomly selected infected individuals; (2) few individuals receive most mosquito bites; (3) primaquine reduces gametocyte carriage duration and might thus be a useful tool if the goal is to have complete interruption of transmission. These observations also generated questions that need to be addressed by future studies: (1) How does human-to-mosquito infectivity relate to heterogeneity in *Anopheles* exposure at different levels (individual and household)? (2) Are estimates of malaria transmission parameters based on infectivity assays similar to estimates based on detailed genetic analysis of parasites [27]? (3) How often are parasites transmitted between households? And is it possible to identify common patterns of malaria connectivity in different settings that would allow rational development of targeted control interventions?

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Appendices

Appendix A, Infectious reservoir of falciparum malaria (past and future)

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Principal Supervisor	Chris Drakeley
Thesis Title	The human infectious reservoir of falciparum malaria

If the Research Paper has previously been published please complete Section B, if not please move to Section C

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Student Signature: Bronner Pamplona Augusto Gonçalves Date: 04/09/2017

Supervisor Signature: Chris Drakeley Date: 04/09/2017

Title: Assessing the infectious reservoir of falciparum malaria: past and future

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Abstract

Renewed interest in malaria eradication has placed greater emphasis on the development of tools to interrupt *Plasmodium* transmission, such as transmission-blocking vaccines. However, effective deployment of such tools will likely depend on improving our understanding of which individuals transmit infections to mosquitoes. To date, only a handful of studies have directly determined the infectiousness of individuals in endemic populations. Here we review these studies and their relative merits. We also highlight factors influencing transmission potential that are not normally considered; the duration of human infectiousness, frequency of sampling by mosquitoes, and variation in vector competence among different mosquito populations. We argue that more comprehensive xenodiagnostic assessments of infectivity are necessary to accurately quantify the infectious reservoir and better target interventions.

Reducing transmission of malaria: the future of elimination programs

The burden of malaria has declined in many endemic settings in Africa and elsewhere [1]. Local malaria elimination is considered achievable with current control approaches in some of these areas when transmission intensity is low and re-introduction unlikely [2, 3]. However, in the majority of endemic areas, operational and technical limitations are likely to hinder the complete interruption of transmission [4, 5]. New or renewed tools aimed specifically at interrupting the spread of *Plasmodium* species from human to mosquito may therefore be critical for future elimination programs [6, 7], especially if transmission efficiency increases as parasite prevalence goes down [8]. Unlike traditional control strategies, which aim to reduce severe morbidity in vulnerable populations, the effectiveness of transmission reducing interventions (TRI) hinges on their coverage of individuals responsible for transmission to mosquitoes regardless of their symptomatic status [9]. Despite the central importance of human infectivity for TRI [10], there have been few direct assessments of human infectivity to mosquitoes. Here we discuss previous studies that aimed to directly assess infectivity at the population level, examine factors necessary to link these controlled transmission experiments with transmission in nature, and advocate the next steps which will provide the key information necessary to better target the human infectious reservoir for malaria.

Quantifying the human infectious reservoir of malaria (1957 – 2014)

In infected humans, the life-cycle of a small portion of the total malaria parasite population culminates in differentiation and maturation into gametocytes (see *Glossary*). When an anopheline mosquito feeds on blood containing mature gametocytes, some may be ingested which then fuse and undergo sporogonic development making the insect infectious to humans. The human component of the infectious reservoir is the proportion of a population that are capable of infecting mosquitoes [11].

In community surveys, data from microscopy typically show that children and infants harbour *Plasmodium* gametocytes more commonly and in greater number than older age groups [12]. Such observations have long galvanised belief among the scientific

community that young individuals represent the main source of infection for mosquitoes [12]. However, in the late 1940s and early 1950s, xenodiagnostic assessments revealed that the presence of gametocytes in blood films was not prerequisite to onward transmission [12-15]. The first population-based assessment of human infectivity to mosquitoes was conducted in rural Liberia, where individuals living in an endemic region were recruited for mosquito feeding experiments regardless of their parasite status [16]. Whilst this study found that young children were much more infectious to mosquitoes, it also showed that when estimates were adjusted for the demographic composition of the population, all age groups were relevant contributors to malaria transmission (**Box 1**). In nearly 60 years since this study a handful similar surveys have been reported (**Tables 1 and 2**). In general, these studies show that whilst young children are consistently more likely to be infectious *per se* [17, 18], the contribution of older age groups is by no means negligible; in previous xenodiagnostic surveys that were able to sample individuals of all ages, individuals >15 years old made up between 21.9 and 40.7% of the total infectious reservoir [16-19]. Several studies have assessed human-mosquito transmission using entomological parameters [20]. However, these studies provide only broad population estimates of infectiousness and they are not able to describe the distribution of the reservoir among different members of the population nor allow an examination of the role of parasite density and infection dynamics.

Box 1. Demography and the malaria infectious reservoir

In previous surveys, the ‘bottom-heavy’ age structure typical of malaria endemic regions has acted to boost the contribution of more infectious children to the total malaria infectious reservoir, while less infectious adults, representing approximately half of most populations, generally contributed to a lesser extent. Recent demographic estimations show that Sub-Saharan African populations are still disproportionately young; approximately 17% and 27% of the population are aged between 0-4 and 5-14 years, respectively (<http://esa.un.org/wpp/>). The influence of demography on patterns of transmission needs to be considered in light of the numerous other factors affecting the likelihood of infectious individuals contributing to mosquito infections. Though infants and children are over-represented in endemic areas they may be the age group least available to and, perhaps, least attractive to mosquitoes (see *Determinants of human transmission potential: the need for data*). Conversely, older children and adolescents may represent a privileged group for human-mosquito transmission; individuals in this age group possess moderate gametocyte densities, approximately double the infectivity of individuals >15 years, lack the severe symptoms that prompt treatment, and appear comparatively vulnerable to mosquito biting (Figure 1).

Women in pregnancy have been observed to attract more than twice as many anophelines as non-pregnant women over short distances [21]. However, such observations must be weighed against behavioural and demographic parameters unique to this group. Census and fertility rate data show that pregnant women comprise a small proportion of African populations. In Zambia, 8.7% of women of child bearing age were pregnant at any one time in 2013, which equates to 2-2.5% of the entire population (<http://www.dhsprogram.com/pubs/pdf/FR304/FR304.pdf>). Pregnant women are also preferentially targeted by net and drug treatment campaigns, so the high attractiveness to mosquitoes of this small proportion of the population may be balanced by interventions.

Table 1. Summary of studies assessing the malaria infectious reservoir where recruitment was conducted without regard for parasite status.^a

Reference	Study location	Local transmission setting	Feeding method	Assay endpoint ^b	Species	Prevalence of infectiousness (%) ^c	Proportional contribution to reservoir (%) ^d				Mosquito infection probability ^e
							1 - 4	5 - 9	10 - 19	≥20	
[19]	Papua New Guinea	Moderate perennial	Direct & Membrane	Oocysts	<i>Anopheles farauti</i>	3.8	22.7	32.9	30.3	14.2	0.013
							1 - 4	5 - 14	≥15	-----	
[16]	Liberia	Intense perennial	Direct	Oocysts	<i>Anopheles gambiae</i>	10.6	40.5	28.4	31.1		0.023
[17]	Kenya	Intense perennial	Direct	Oocysts & CSP ELISA	<i>Anopheles gambiae</i>	10.1	23.2	36.1	40.7		0.010
[77]	Burkina Faso	Intense seasonal	Membrane	Oocysts	<i>Anopheles gambiae</i>	48.0	ND ^f	39.7	60.3		0.055
[18]	Cameroon (Bondi)	Intense seasonal	Direct	Oocysts	<i>Anopheles gambiae</i>	7.4	28.8	49.2	21.9		0.024
[18]	Cameroon (Mengang)	Intense seasonal	Direct	Oocysts	<i>Anopheles gambiae</i>	8.2	34.7	33.9	31.4		0.011
[32]	Senegal	Low, unstable	Direct	Oocysts	<i>Anopheles arabiensis</i>	8.7	ND ^f	51.8	48.2		0.002

^a Only studies that have utilised demographic and mosquito feeding data to characterise the human infectious reservoir of *Plasmodium* in endemic populations were included. All studies were conducted in areas where *Plasmodium falciparum* and non-*falciparum* species co-circulate, so the outcome of feeding assays cannot be attributed unambiguously to *falciparum* malaria. One additional study not included in the table utilised population and xenodiagnostic data to quantify the malaria infectious reservoir in Sri Lanka [79]. This study recruited individuals with incident malaria over a 17 month period, the majority of which were *Plasmodium vivax*. The study design and analysis was suitably different as to exclude the possibility of direct comparison with the other studies presented in the table. ^b Assay endpoint was either 1. Oocysts counted on the mosquito midgut by microscopy, or 2. Oocysts counted by microscopy or positivity of remaining live mosquitoes at the end of the experiment (day 15) post infection) in the circumsporozoite protein ELISA [17]. ^d Proportional contribution to the reservoir presents the proportional contributions of three or four age groups to population-adjusted reservoir, with age groupings combined where the data in the original publication allowed. ^e Mosquito infection probability corresponds to the probability that a mosquito biting a random individual in a population will develop infection. This measure was either presented as in the published data, or was calculated by combining demographic data, age-specific prevalence of infectiousness and age-specific mosquito infection rates during infective membrane feeding experiments. ^f ND: Studies failed to perform mosquito feeds on individuals <5 years of age. Data has been adjusted to represent the infectious reservoir of individuals >5 years only.

Table 2. Summary of studies assessing the malaria infectious reservoir that recruited only gametocyte positive individuals for feeding assays.^a

Reference	Study location	Local transmission setting	Feeding method	Assay endpoint ^b	Species	Prevalence of infectiousness (%) ^c	Proportional contribution to reservoir (%) ^d				Mosquito infection probability ^e
							1 - 4	5 - 9	10 - 19	≥20	
[11]	The Gambia	Intense seasonal	Membrane	Oocysts	<i>Anopheles gambiae</i>	5.6	17.5	21.7	22.2	37.9	0.015
[11]	Tanzania	Intense perennial	Membrane	Oocysts	<i>Anopheles gambiae s.l.</i>	3.2	28.4	23.1	14.2	34.0	0.007
[19]	Papua New Guinea ^f	Moderate perennial	Direct & Membrane	Oocysts	<i>Anopheles farauti</i>	3.6 (1.4)	48.0	12.4	24.0	15.5	0.012 (0.004)

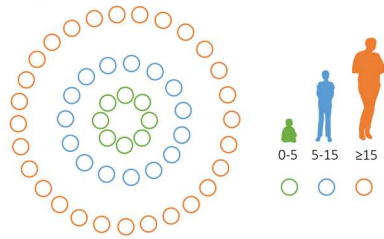
^aOnly studies that have utilised demographic and mosquito feeding data to characterise the human infectious reservoir of Plasmodium in endemic populations were included. All studies were conducted in areas where *Plasmodium falciparum* and non-falciparum species co-circulate, so the outcome of feeding assays cannot be attributed unambiguously to falciparum malaria. ^bAssay endpoint was oocysts counted on the mosquito midgut by microscopy. ^cPrevalence of infectiousness was calculated as the combined prevalence of individuals infectious to ≥ 1 mosquito in each age category, adjusted for proportion of the population made up by the age groups in the total population. ^dProportional contribution to the reservoir presents the proportional contributions of three or four age groups to population-adjusted reservoir, with age groupings combined where the data in the original publication allowed. ^eMosquito infection probability corresponds to the probability that a mosquito biting a random individual in a population will develop infection. This measure was either presented as in the published data, or was calculated by combining demographic data, age-specific prevalence of infectiousness and age-specific mosquito infection rates during infective membrane feeding experiments. ^fFor this study, all species and *P. falciparum*-specific (within parenthesis) estimates are presented.

Ultimately, determining the infectiousness of different age groups relative to their representation in the population is just one necessary step in characterising the infectious reservoir. Contribution to transmission is influenced not only by intrinsic parasite and human determinants of infectivity, reflected in the outcome of mosquito feeding assays, but by a variety of additional factors that make the capacity to infect mosquitoes in controlled experiments distinct from the probability of onward transmission in nature (*e.g.* human exposure to mosquito biting) (**Figure 1**). These factors are not commonly taken into account and are not uniform across time, space, or between individuals of different age. To more accurately reflect transmission in its epidemiological context, these elements need to be considered and incorporated into future assessments.

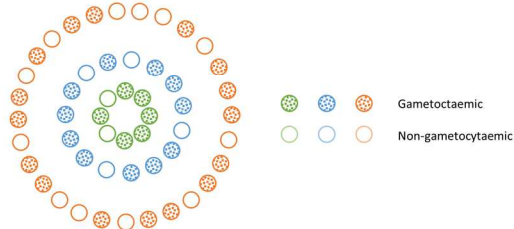
Figure 1. Age contributions to the infectious reservoir.

A. Individuals in the figure are represented by circles in three age groups; <5, 5-15, and >15 years. The abundance of individuals in each group reflects a simplified population age-structure in sub-Saharan Africa, as described in Box 1 and in the studies detailed in Table 1 and 2 (<5=15%, 5-15=30%, >15=55%) (<http://esa.un.org/wpp/>). **B.** Speckling within circles represents the presence of *Plasmodium falciparum* gametocytes detectable using quantitative-nucleic acid sequence-based amplification (QT-NASBA); age-specific prevalence presented here were taken from an area of intense seasonal transmission [87]. **C.** Solid filled circles represent who might infect mosquitoes in a mosquito feeding assay. Some (or most) gametocytaemic individuals may not be infectious for a variety of reasons, including gametocyte density, maturity, recent treatment, and transmission blocking immunity. Infectiousness prevalence for the three age groups (<5=25%, 5-15=13%, >15=7%) is based on surveys described in Table 1 [16-18]. Pie chart 1 demonstrates the proportional contribution of each age group to the total population infectious reservoir. Demographic adjustment is not necessary as this example directly represents the age-composition of a typical population. Of the theoretical total infectious population (9.8% [5/51 individuals]), individuals in the age groups <5, 5-15 and >15 years account for 40%, 40% and 20% of the reservoir respectively. **D.** Equally exposed individuals of different ages are differently attractive to mosquito biting, partly due to differences in body surface area. To reflect this, individual circles were given a surface area proportional to the average body surface area of individuals in the three age groups (<5=0.4m², 5-15=1.2m², >15=1.6m²; Ratio of surface area 1:3:4), using data from Port *et al.* showing that body surface area correlated positively with mosquito biting rate [45]. Pie chart 2 illustrates the potential effect of increased mosquito sampling due to body surface area in each age group on the infectious reservoir. Finally, to account for some of the age-related differences in exposure intensity, the percentage bed net use among individuals in broadly similar age groups from an area of seasonal transmission in Western Kenya (Coverage: <5=42%, 6-17=26%, >17=57.7%; Ratio of exposure 1:1.2:0.7) was used to adjust the surface area weighted contributions to the infectious reservoir (Pie chart 3) [88].

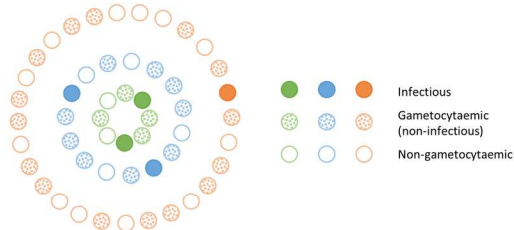
A. Age-stratified population



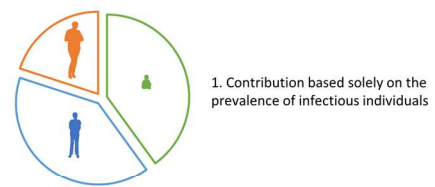
B. Proportion of individuals with gametocytes



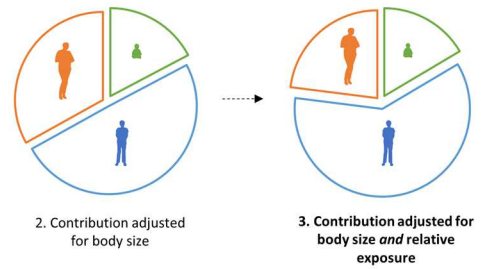
C. Proportion of individuals infective to mosquitoes



Proportional contribution to total infectious reservoir by age



D. Individuals adjusted for surface area/mosquito biting frequency



Determinants of human transmission potential: the need for data

Duration and dynamics of infectivity

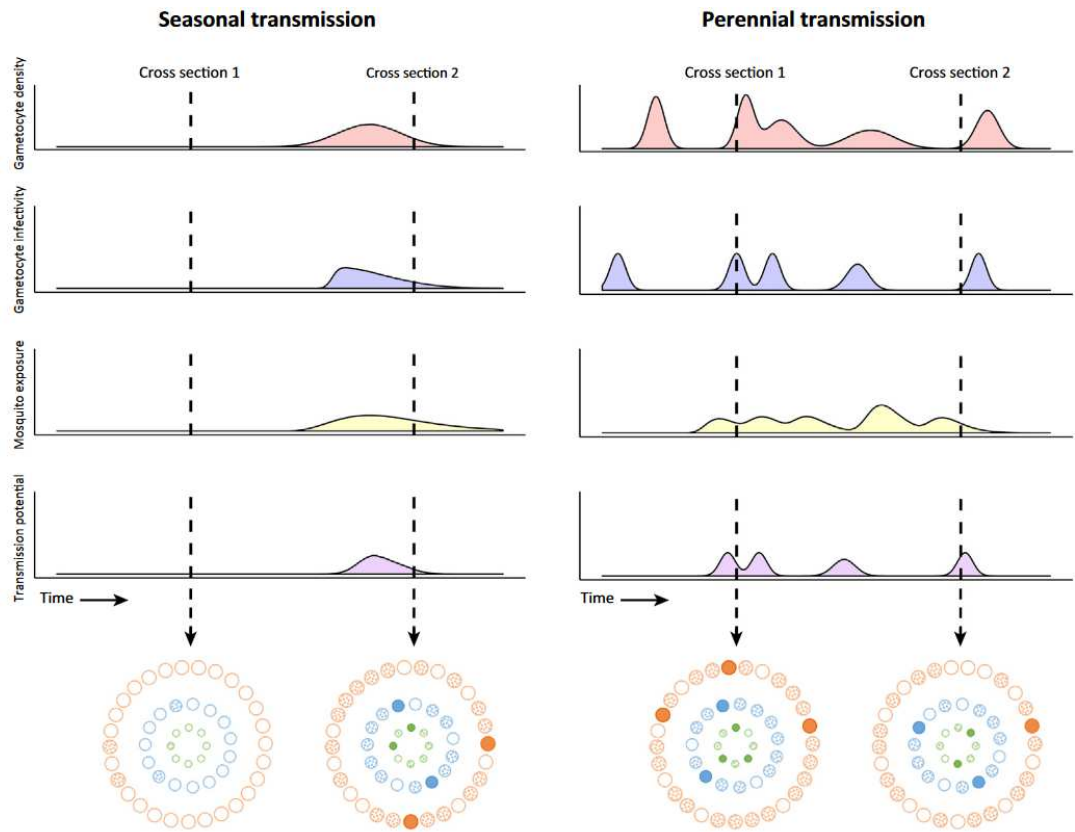
The likelihood of transmission to *Anopheles* mosquitoes is determined primarily by gametocyte density, fitness and their circulation time. The non-linear relationship between gametocyte density and mosquito infection risk has been described several times [21-23]. Yet, because of the relatively high proportion of low density parasitaemias and gametocytaemias in natural infections, there is increasing focus on how important these often sub-patent infections are for the maintenance of transmission [24, 25]. While the limited sensitivity of microscopy to detect gametocytes is well established [21], many claims of submicroscopic gametocyte densities contributing to transmission are based on microscopy-positive asexual parasite carriers with accompanying submicroscopic gametocyte densities. This is an important difference when considering how current diagnostics may capture the human infectious reservoir [26, 27]; infections with submicroscopic gametocytes that accompany patent asexual parasites are detectable by conventional diagnostics whilst infections with no microscopically detectable parasite stages require more sensitive diagnostics [28].

Because of their cross-sectional nature, previous xenodiagnostic surveys have not formally examined the duration of infectiousness, another key factor influencing transmission potential. Data from malaria therapy studies suggest infectiousness of an individual infection can last for many months [29] however it is difficult to extrapolate the observations from a limited age range of malaria naïve to endemic populations. Broadly, in endemic settings, children (possessing limited immunity) are likely to have more acute, higher density infections that often require treatment [30]. This may result in cross sectional surveys missing recently treated infections and identifying only a limited number of highly infectious children without recent treatment. Conversely, adults and older children (often semi-immune, and presenting few clinical symptoms) would have longer, chronic infections, which could result in longer infectious periods, balanced by lower infectivity at individual time points [31]. These patterns of infection are likely to differ with transmission intensity. In areas of intense malaria transmission children may have repeated malaria infections with relatively high gametocyte densities and a high likelihood of malaria transmission (**Figure 2**). In areas with lower

transmission, including areas that have reduced transmission in the context of an elimination program, infection duration will likely have a more significant influence on the infectious reservoir. At present only one study has directly assessed population level infectiousness in an area of low malaria transmission [32]. Because of the utility of these data to control and elimination programs, further studies assessing the infectious reservoir in areas of low transmission would be advantageous.

Figure 2. Cross-sectional surveys and infectious reservoir dynamics.

Data represent hypothetical annual transmission measures in two different endemic settings of seasonal and perennial malaria transmission. The dashed line corresponds to the timing of cross-sectional xenodiagnostic surveys. The population plots underneath the main charts reflect hypothetical population level infectiousness, as would be measured in direct assessments of the reservoir. Individuals in the plots are represented by circles in three age groups; <5, 5-15, and >15 years. The abundance of individuals in each group reflects a simplified population age-structure in sub-Saharan Africa, as described in Box 1 and in the studies detailed in Table 1 and 2 (<5=15%, 5-15=30%, >15=55%) (<http://esa.un.org/wpp/>). Speckling within circles represents the presence of *Plasmodium falciparum* gametocytes; age-specific prevalence is hypothetical. Solid filled circles represent who might infect mosquitoes in a mosquito feeding assay; infectiousness prevalence for the three age groups is hypothetical. To reflect differences in body size and potentially attractiveness to mosquitoes, individual circles were given a surface area proportional to the average body surface area of individuals in the three age groups (<5=0.4m², 5-15=1.2m², >15=1.6m²; Ratio of surface area 1:3:4), using data from Port *et al.* showing that body surface area correlated positively with mosquito biting rate [45]. The combined surface area of individuals in the plots can be interpreted broadly as the contribution each group makes to the total infectious reservoir. Combined, the figure illustrates that the total infectious reservoir and the proportional contribution of differently aged individuals are likely to vary, potentially significantly, over time in response to the dynamicity of numerous factors. Though variations in transmission potential would probably be clearest between surveys where transmission is seasonal, age-specific contributions may vary with time in areas where transmission is intense and perennial.



In an individual infection, infectiousness per gametocyte may be higher at the beginning of an infection than at the end because of changes in parasite competency [33], sex ratio [34, 35] and possibly the development of sexual stage immune responses [36-38]. Transmission-blocking immunity is boosted by recent exposure to gametocytes [37] and may reduce infectiousness during the course of infections that last several weeks or months. Superinfecting clones influence parasite growth dynamics including total gametocyte biomass [39], and multiplicity of infection could in itself be a determinant of transmissibility [40]. This combination of factors might also indicate that infectiousness of one infection may not reflect the infectiousness of subsequent infections (when multiplicity of infection and transmission blocking immunity may differ), suggesting greater complexity than that estimated from a single time point during the transmission season.

Mosquito exposure

Artificial feeding experiments in xenodiagnostic surveys have tended to use fixed numbers of mosquitoes, improving the precision of estimates of infectiousness by maximising mosquito sample sizes. However, in any endemic area mosquito biting rates of humans vary considerably. As an example, a recent study in Uganda found biting rates varied from 270 to 7399 bites/year between study sites, with each site showing distinct seasonal fluctuations [41]. The transmission potential of infectious individuals will be determined by the rate at which they are sampled by local mosquitoes and the competence of these vectors. Where vector densities and survival rates are low the number of mosquitoes that become infected and subsequently transmit their infections will be very low even if there are infectious individuals in the human population. Although essential to accurately characterize individual-specific contributions to transmission, these entomological parameters have never been measured in conjunction with xenodiagnostic surveys.

Attractiveness to mosquitoes

In the early 1950s, Muirhead-Thomson observed that Jamaican adults attracted more *Anopheles* mosquitoes than equally exposed children [42]. The conclusions of this study are supported by later reports in which wild *Anopheles* were allowed to feed on

unprotected humans under observation [43, 44], or ABO blood typing allowed the identification of blood meal origin [45], though some studies show more random biting selection [46]. Carnevale *et al* near Brazzaville, in the Republic of Congo, described the biting behaviours of 6500 mosquitoes on 24 individuals of all ages [43]. *Anopheles* biting increased with age, with individuals >20 years old being bitten three times more than < 2 year olds. Port *et al.* were able to ascribe this age-related biting pattern to increased body weight and surface area [45]. In addition to increased surface area, body temperature and chemical cues change with age and physiological maturity and may be associated with attractiveness [47-49]. This might also help explain why pregnant women are thought to be more attractive to mosquitoes than non-pregnant women [50]. Muirhead-Thomson also observed that infants reacted more actively in sleep to the probing of mosquitoes than adults, and that mosquitoes were less likely to feed on infants even after alighting on their skin [42]. Data are less robust on the influence of *Plasmodium* infection on mosquito attractiveness. Reports from animal models and studies on a small number of humans infected with *P. falciparum* suggest that the presence of gametocytes may increase attractiveness to anopheline mosquitoes [51, 52], possibly due to the production of attractive volatiles [53]. It is unclear if the small biomass of gametocytes could have specific systemic effects that would increase the attractiveness of children (harbouring higher gametocyte densities), and if this might counteract the reduced attraction associated with their smaller surface area.

Availability to mosquitoes

However attractive and infectious the individual, they must be available to mosquitoes for feeding and subsequent transmission to occur. This availability is shaped primarily by human social and behavioural practices, including use of mosquito nets and sleeping patterns.

Age-specific net use depends on the setting and extent of ownership, but in areas with good coverage appears highest among young children (<5 years), and lowest among older children and adolescents (5-15 years) [54-57]. In addition to bed-net's community-wide effect on transmission, they may also reduce an individual's chance of infecting mosquitoes and divert vectors to other bloodmeal sources. In the Gambia, the introduction of insecticide-treated nets (ITN) protected children against malaria and diverted mosquito bites to other hosts (animals or adults) [58]. Net distribution has also been linked to changes in vector behaviour and species composition;

Anopheles gambiae and *Anopheles funestus* have showed signs of responsive exophagy [59], and there are observations that peak biting in these species may shift to the early evening and morning [60, 61]. Net-related reductions in *A. gambiae* s.s. populations have also been linked to increasingly dominant roles for *A. funestus* [62] and *Anopheles arabiensis* [63].

These elements together with sleeping times and time spent indoors or outdoors at night determine availability to different mosquito populations. Children might be expected to sleep earlier than adults, but the protection afforded by long periods of time under bed-nets depends on when local vectors are most active. Data on time spent indoors, time spent under nets, and on the biting activity of local vectors can be combined to determine the true protective efficacy of bed nets by age [64]. This is an informative composite measure, which could be used to estimate age-specific patterns of mosquito exposure.

Effectively delineating the interaction of mosquito exposure and subsequent malaria transmission is difficult. Individuals identified as infectious in xenodiagnostic surveys must have been exposed to malaria vectors at some stage, but heterogeneity in attractiveness and the degree of exposure should still significantly influence their relative contribution to the infectious reservoir. Surveys quantifying mosquito exposure and infectiousness in the same population could provide more realistic estimates of age-specific contributions to transmission.

Heterogeneous Mosquito Populations

Although several vector species might contribute to local transmission in endemic areas, most xenodiagnostic surveys have been performed using *A. gambiae* s.s. (Tables 1 and 2), the majority of which were colony reared. Evidence is limited on whether infectiousness to *A. gambiae* during membrane or skin feeding assays corresponds to infectivity to other vector species. Two major malaria vectors, *A. funestus* and *A. arabiensis*, are distributed throughout Sub-Saharan Africa, often co-existing with *A. gambiae* s.s. [65]. The highly anthropophilic biting preferences of *A. funestus* [66], its high longevity [67] and sporozoite rate [62, 68] (higher than *A. gambiae* in some endemic areas) indicate that a non-negligible part of malaria transmission might be

sustained by this vector where it is present. Conversely, *A. arabiensis* is considered more exophilic and exophagic; in Burkina Faso, *A. arabiensis* was as susceptible to infection as *A. gambiae* s.s. in feeding assays [69], while in Senegal, lower infection rates were observed in membrane feeding assays with *A. arabiensis* compared to *A. gambiae* s.s. [70]. The recent description of cryptic malaria vectors [71, 72] and the conflicting results on the differential susceptibility of *A. gambiae* s.s. and *Anopheles coluzzi* [69, 70] (previously the molecular forms S and M of *A. gambiae*, respectively [73]), further illustrate the need to quantify transmission efficiency against the background of diverse vector populations.

Considerations for future studies

To more rigorously assess the human infectious reservoir using direct xenodiagnostic surveys, several methodological issues require consideration. In this section, we suggest suitable inclusion criteria and methods for measuring or predicting individual level mosquito exposure. Other important factors that are discussed include the type of feeding assay, measures of infectiousness, and general study design.

Inclusion criteria for direct assessments of the infectious reservoir

There is abundant evidence that microscopy is insufficiently sensitive to detect low densities of asexual parasites and gametocytes [15, 19, 74-78]. Studies including only individuals with patent gametocytes [11, 19, 79] are likely to have underestimated the proportion of populations capable of infecting mosquitoes, and biased measures of mosquito infection probability (Table 2). Since low density gametocyte carriage is prevalent in many endemic areas [25] and they may represent a major source of secondary mosquito infections [22, 80, 81], studies aiming to characterize the human infectious reservoir should not have gametocyte positivity by microscopy as an inclusion criterion.

Defining infectiousness at the population level

The simplest method for quantifying the infectious reservoir combines the prevalence of people in different age groups infecting at least one mosquito, and the proportion

of those groups in the total population [16, 77]. Alternatively, population level infectiousness may be presented as mosquito infection probability (K); the likelihood of a mosquito becoming infected after feeding on any member of the population, calculated as the age-adjusted mosquito infection rate. Although the former metric has value for efforts that aim to identify or target all individuals who contribute to onward malaria transmission, the latter (mosquito infection probability) makes estimates more comparable between studies, where the number of mosquitoes used in feeding assays may differ widely [18, 19], and captures variations in infectiousness intensity in different age groups that may not be apparent when reporting only infectiousness prevalence.

Assay methodology

There are two types of mosquito infection assay that have been used in previous surveys: direct skin feeding and membrane feeding assays (Table 1 & 2). Direct feeds better reflect natural infection and avoid some of the technical challenges that limit the wide-scale use of membrane feeding assays (*e.g.* adapting mosquito colonies to membrane feeding, preventing gametocyte activation between bleeding and feeding and a potential effect of anticoagulants) [82, 83]. Although their use is currently hindered by ethical concerns that have precluded their use in repeated assessments of mosquito infection from young children [82], further direct comparisons of xenodiagnostic surveys using skin and membrane feeding experiments would be highly valuable to parameterise the extent to which the latter reflects the natural situation. Unless direct skin feeding is acceptable in all age groups and with sufficient mosquito numbers to allow precise estimates of infection, studies aiming to characterise the full infectious reservoir in endemic populations should do so using direct membrane feeding assays using locally relevant malaria vectors.

Whether feeding assays measure infection by detection of oocysts in the mosquito gut or sporozoites in the salivary glands (**Tables 1 and 2**) should make little difference to the comparability of future studies, as even in low intensity infections (such as those occurring commonly in nature) there is a close correlation between oocyst prevalence and the prevalence of subsequent salivary gland infection [84]. Salivary gland dissections are technically even more demanding than midgut dissections, will result in higher mosquito mortality between the day of feeding and the day sporozoites are

first detectable and impose additional safety measures for insectaries that need to be equipped for holding mosquitoes that form a biohazard to insectary personnel. We therefore suggest that detection of *Plasmodium* oocysts is the most viable measure of mosquito infection.

Measuring mosquito exposure

As outlined earlier, the actual number of mosquito bites an individual receives is influenced by their physical characteristics and behaviour [42, 45]. There is also spatial heterogeneity in exposure, with individuals living in households with higher vector densities (*e.g.* houses nearer to breeding sites) receiving more bites and having greater risk of disease than those in households where vector density is lower [85]. Infectious reservoir assessments should ideally use a direct approach to quantify mosquito biting rates, simultaneously accounting for the availability and attractiveness of human hosts to mosquitoes: collection of mosquitoes from households and identification of blood meal sources by DNA fingerprinting would provide reliable estimates of true exposure to mosquito bites. A recent study in Peru used this approach to show that adults were more often bitten by *Aedes aegypti* mosquitoes compared to children living in the same houses [86]. We suggest that in areas where transmission is dominated by indoor biting mosquitoes, the rate of secondary mosquito infections could be calculated by multiplying individual-specific mosquito biting rates (determined experimentally using the above methods) by infectiousness (the probability that a mosquito develops infection after feeding, determined with mosquito feeding assays). This measure would be informative as it accounts for all major factors influencing transmission potential. An obvious problem with this approach is that only endophilic mosquitoes are sampled and there are currently no standardized sampling strategies for outdoor biting vectors.

Where direct assessments are operationally impossible, or made unreliable by vector behaviour, we suggest that daily mosquito biting rates could be estimated for each study subject by collating mosquito (*e.g.* abundance, estimated indoor and outdoor biting rate), demographic (*e.g.* age, body surface area and attractiveness, household conditions, distance to breeding sites, number of people living in the same house/room) and behavioural (*e.g.* sleeping times, net use) data, so that a rate of

secondary mosquito infections could be broadly estimated by combination with experimentally-determined infectiousness. Figure 1 is a simplified and hypothetical representation of the potential influence of some of these factors on the contribution of different age groups to the infectious reservoir.

Longitudinal studies with mosquito exposure estimation

Longitudinal studies with frequent follow-up would be valuable not only to estimate the duration of infectiousness in different age groups but also to assess whether some individuals or groups of individuals are infectious throughout a transmission season. This approach would help determine if transmissibility is associated with clusters of malaria higher infection incidence, *i.e.* where mosquito-to-human transmission is high. The more complete interpretation of such assessments could be made with concurrent mosquito biting rates.

Concluding remarks

We acknowledge that there are several malaria endemic settings in which progress toward elimination has been made in the absence of detailed local knowledge of the infectious reservoir. However, malaria remains a threat in many countries, particularly in Africa, and resurgence due to natural receptivity for transmission, drug and insecticide resistance means that a better understanding of who within a community infects mosquitoes can only benefit malaria control.

The complexity of factors influencing malaria transmission means that studies aiming to fully understand how this occurs is challenging. Previous xenodiagnostic surveys provided valuable insights into malaria transmission epidemiology, but these studies need to be extended to simultaneously evaluate additional key factors. Moreover, the longitudinal nature of infectiousness during natural malaria infections needs to be considered in future studies, as this will influence the contribution of different age groups to transmission.

While human and parasite factors, including the intensity and duration of gametocytaemia, affect infectiousness at the individual level, entomological factors dictate the transmission potential of each individual and the infectious reservoir as

whole. Therefore, mosquito exposure assessments need to be an integral part of xenodiagnostic surveys if we are to effectively target the reservoir of infection.

Box 2. Outstanding questions

If and how does human population infectiousness to mosquitoes differ in areas of low malaria transmission? Are traditionally low endemic settings different from those under epidemiological transition (i.e. those settings moving from moderate to low transmission as a result of heightened control activities)?

What is the duration of infectiousness of individuals to mosquitoes? Are certain people infectious for longer periods and, if so, what are their characteristics?

How do fluctuations in mosquito densities and feeding rates affect human infectiousness?

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Appendix B, Pregnancy and malaria elimination

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Student	Bronner Pamplona Augusto Gonçalves
Principal Supervisor	Chris Drakeley
Thesis Title	The human infectious reservoir of falciparum malaria

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Student Signature: Bronner Pamplona Augusto Gonçalves Date: 04/09/2017

Supervisor Signature: Chris Drakeley Date: 04/09/2017

Title: Pregnant women: an overlooked asset to falciparum malaria elimination campaigns?

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Abstract

Community chemotherapy campaigns to reduce malaria transmission often exclude pregnant women due to safety concerns related to the drugs. However, pregnant women might represent an important source of human-to-mosquito infection due to frequent parasite carriage with higher densities of parasites (often detectable by microscopy), attractiveness to mosquitoes and modified sleeping behaviour. Accumulating evidence of the safety of artemisinin-based combination therapies for treatment of malaria during gestation suggests that malaria elimination programmes should reconsider this exclusion. Including pregnant women will increase intervention coverage and impact, and may thereby accelerate progress towards the desired endpoint (e.g. elimination) or increase the chances of success. Studies assessing infectiousness of pregnant women and gametocyte dynamics during different trimesters of pregnancy will be valuable to support the planning of community treatment campaigns.

Community chemotherapy campaigns and excluded populations

The World Health Organization (WHO) recently recommended mass drug administration (MDA) to accelerate *Plasmodium falciparum* elimination in areas approaching zero endemic transmission and in the Greater Mekong area to prevent global spread of artemisinin resistant parasites [1]. Community chemotherapy approaches, including MDA, have been deployed in a number of settings [2] with varying levels of transmission and, not surprisingly, these studies indicate that high population treatment coverage (>80%) that is sustained over repeated rounds is a major determinant of success [3]. Indeed, it is the capacity of these community-based treatment campaigns to clear a high proportion of asymptomatic malaria infections, especially those with higher parasite densities, which are more infectious to mosquitoes, and provide a period of effective malaria prophylaxis that will determine whether transmission will be eventually interrupted. Individuals who are not 'covered' or who are missed by drug administration typically include those absent due to travel or work, those with contraindication to the drug, those who are seriously ill and those who do not consent to participate. The limited data from previous studies suggest that both absenteeism and ineligibility are relevant factors in determining non-participation [4, 5]. The malaria infection prevalence of these non-participating individuals at the time of the intervention, and therefore their contribution to transmission to mosquitoes and the extent to which they adversely affect the outcome of MDA, are likely to be context-dependent.

Young children and pregnant women are easily accessible to malaria control programmes, but are frequently excluded from MDA target populations (for example, [5, 6] and <https://clinicaltrials.gov/ct2/show/NCT02914145>) due to safety concerns related to the drugs being administered. The impact of excluding young children on residual malaria transmission will be dependent on the age cut-off used by control programmes to define eligibility: for example, the non-inclusion of infants aged 6 months or younger will generally represent around 1.5 – 2.0% of the population. This is expected to have only a minor effect on post-MDA transmission as these children are protected against malaria [7] through a variety of mechanisms (e.g. maternal antibodies, foetal haemoglobin [8], etc). They are also bitten by mosquitoes less often than adults [9, 10] due to their smaller body size [11] and the fact that they are more

likely to sleep under bed nets compared to older individuals living in the same household [12]. The contribution of pregnant women to malaria transmission, on the other hand, has not been evaluated and it is not clear whether additional efforts may need to be made to include them in future community-level treatment approaches.

In this opinion article, we consider epidemiological and entomological evidence that pregnant women contribute to *P. falciparum* malaria transmission, use simple calculations to argue that they represent a non-negligible part of the infectious reservoir, discuss the impact of malaria prevention strategies on their transmission potential, and suggest community-based diagnostics of high-density infections in pregnant women to improve both MDA effectiveness and maternal health.

Malaria transmission during pregnancy

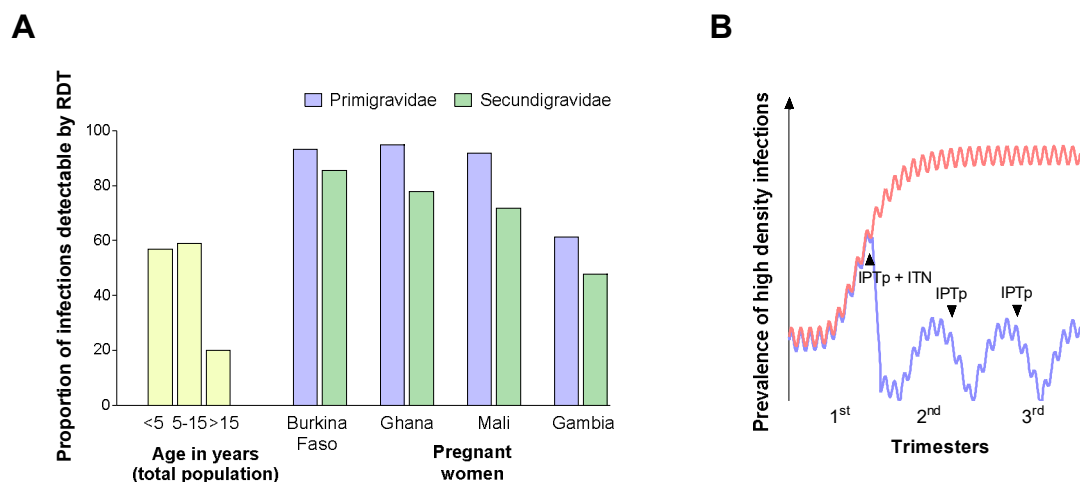
During pregnancy, women immune to high-density falciparum infections through previous exposure become susceptible again due to the abundance of the parasite adhesion receptor chondroitin sulfate A (CSA) in the placenta [13], which allows asexual stage parasites to sequester and develop in the intervillous space. This can cause high parasite densities resulting in morbidity for mother, foetus and infant [14]. While in areas with low malaria transmission women of all parities are at risk of developing these high-density placental infections, where exposure to malaria parasites is more frequent, women acquire immunity that prevents adhesion of CSA-binding parasites with successive pregnancies [15, 16], the average density of placental infections decreases with gravidity, and primigravidae are at highest risk. In addition to the deleterious effects to the mother and her offspring, the relatively high *P. falciparum* parasite prevalence and densities, both in the placenta and in the peripheral circulation [17], compared to non-pregnant adults implies that pregnant women might be an important source of malaria infection to mosquitoes. These high asexual stage parasite densities could generate high numbers of circulating gametocytes (the stage of the parasite life cycle that infects Anopheline mosquitoes) in pregnant women. As previously hypothesised [18], pregnancy-associated changes in the bone marrow [19], an organ where immature gametocytes develop [20, 21], could also potentially influence gametocyte levels. However, few epidemiological studies have assessed the presence of gametocytes during pregnancy [18, 22, 23]. In a

study in a peri-urban area in Malawi [18], the prevalence of sexual stage parasites in pregnant women at their first antenatal care visit was 4.9% whilst 20% of pregnant women with asexual stage parasites detected by microscopy had patent gametocytes. In the same study, gametocytaemia at enrolment was frequently associated (80%) with placental haemozoin deposition at delivery. Although these observations suggest that in the absence of treatment placental infections might result in chronic high density (patent) gametocyte carriage, a comparison with non-pregnant adults living in the same community and consequently exposed to the same infection risk would be necessary to assess whether pregnant women are more frequently gametocytaemic and have a higher gametocyte commitment during infections. A more comprehensive quantification of the importance of pregnancy-associated malaria to transmission could be achieved by the measurement of gametocytes by both microscopy and molecular diagnostic methods [24, 25] during antenatal care visits. Ideally this would be supported by mosquito feeding assays [26], a method that allows assessment of malaria infectivity, in a selection of individuals to estimate the association between gametocytaemia and mosquito infection rates in this specific subpopulation.

Previous studies [27-30] estimating the contribution of different age groups to the infectious reservoir of *P. falciparum* malaria using experimental mosquito infection assays (reviewed in [31]) did not specifically report on the infectiousness of pregnant women. We can, however, indirectly estimate the proportion of potentially infectious individuals that pregnant women represent by examining the percentage of women that are pregnant, and their likely infectiousness prevalence. Data on the proportion of the female population that is pregnant are available for many malaria endemic countries, and in Ghana, for example, 7.1% of women aged between 15 and 49 years were pregnant at the time of a recent demographic survey (dhsprogram.com/pubs/pdf/FR307/FR307.pdf), which corresponds to 1.7% of the total population. Although malaria infectivity has not been assessed during pregnancy, transmission studies have determined infectiousness prevalence (i.e., the percentage of individuals able to infect at least one mosquito in membrane feeding experiments) in different age groups. Based on differences in parasite density between pregnant women and non-pregnant adults, which is higher in the former, it is expected that the proportion of pregnant women that are infectious will be more similar to the proportion of children who are infectious than to the proportion of non-pregnant adults that can

infect mosquitoes. **Figure 1** illustrates this difference in parasite burden during established infections: pregnant women, especially primigravidae, with falciparum infection detected by molecular methods often had parasites detected by standard malaria rapid diagnostic tests (RDTs), which have a relatively high limit of detection of 100 to 200 parasites per μL [32]. Assuming two different scenarios of infectiousness during pregnancy (in one scenario, infectiousness prevalence in pregnant women is similar to that in schoolchildren; in the other, pregnant women and non-pregnant adults have similar infectiousness prevalence), pregnant women could represent 0.8 – 2.9% of the infectious population (**Box 1**), and up to 23.9% of all infectious individuals immediately after an MDA round with 90% coverage of the non-pregnant population, when transmission might resurge due to infected untargeted individuals. These are only rough estimates as detailed data on gametocyte carriage and infectiousness in pregnant women would be required to develop mathematical models of malaria transmission potential during pregnancy and to estimate the impact of repeated MDA rounds on the contribution of pregnant women to residual transmission. To a great degree, these estimates are also likely to vary according to transmission intensity, which determines susceptibility to infection (and subsequent infectiousness) in both pregnant women and non-pregnant individuals.

Figure 1. Relative parasite burden in pregnant women, non-pregnant adults and children and effect of preventive measures on high-density parasitaemia during pregnancy. In this figure, high-density infections are those detectable by malaria rapid diagnostic tests (RDT+). In A, the proportion of infections detected by PCR (PCR+) that are also RDT+ (y-axis) is presented: baseline data at enrolment from a multicentre randomised trial were used to estimate this proportion for pregnant women (re-analysis of data from [55]); for comparison, age-specific estimates by Wu and colleagues for areas with parasite prevalence higher than 20% are also presented [32]. In B, the prevalence of high-density infections (y-axis) throughout pregnancy (x-axis) is illustrated for pregnant women receiving (blue line) or not receiving (red line) preventive measures. As the pregnancy progresses from the first to the second trimester, infections that were initially submicroscopic reach levels that can be detected by RDTs in the absence of interventions. In the presence of interventions the prevalence of RDT+ infections remains lower and the detectability fluctuates around the baseline prevalence of parasites by RDT in the adult population. IPTp, intermittent preventive treatment; ITN, insecticide treated nets.



Box 1. Pregnancy and the malaria transmission reservoir.

We estimated the contribution of pregnant women to the pool of infectious individuals and of mosquito infections. Demographic data from Ghana (The DHS Program, dhsprogram.com/pubs/pdf/FR307/FR307.pdf) were used (**Figure I**, panel A). Contributions to the population of infective individuals (**Figure I**, panel B; the radius of this graph is proportional to the age-adjusted infectiousness prevalence in the population) were calculated using the following equation, which is similar to formulas used by previous xenodiagnostic studies [27, 30]:

$$[1] \quad C_i = d_i t_i / \sum_i d_i t_i$$

C_i is the proportion of all infectious individuals that each group i represents; and d_i relates to the demographic structure (percentage of individuals in each age group). Since individuals with subpatent parasite levels can infect mosquitoes and most xenodiagnostic studies performed thus far did not present infectivity data by microscopy status, we used group-specific transmissibility prevalence (t_i) in our calculations. For panels B and C, age-specific infectivity data from Bonnet and colleagues (2003) were used [30], and we assumed that pregnant women are as infectious as schoolchildren (infectiousness prevalence of 11.4%). Under these assumptions, estimated C_i values for children younger than 5 years, children aged between 5 – 15 years, non-pregnant adults and pregnant women were 0.26, 0.46, 0.25 and 0.03.

To estimate the contribution of different groups to the reservoir of infected mosquitoes (**Figure I**, panel C; a hypothetical population of 100 infected mosquitoes is represented), we defined:

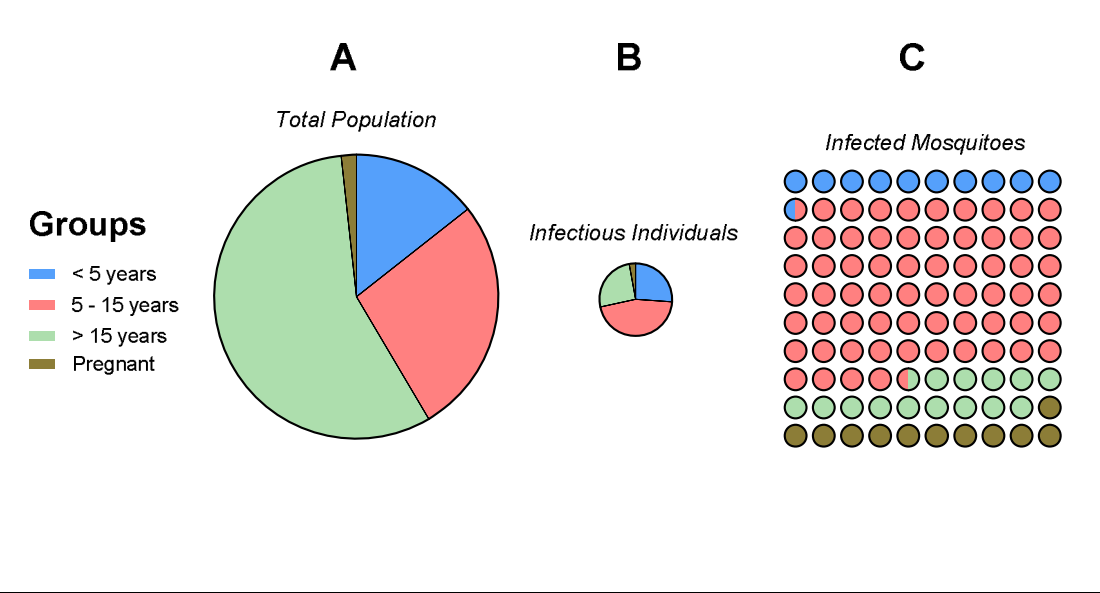
$$[2] \quad M_i = b_i p_i / \sum_i b_i p_i$$

$$[3] \quad b_i = d_i a_i / \sum_i d_i a_i$$

We assumed that the proportion of infected mosquitoes in a given area acquiring their infection from group i (M_i) depends on the proportion of the total number of mosquito bites on individuals from each group (b_i ; a_i representing relative attractiveness to mosquitoes) and the probability (p_i) of mosquito infection after feeding on an individual from group i , itself dependent on the infectiousness prevalence and mosquito infection rates in positive experimental transmission assays. In these

calculations, we assumed that a_i increases with age, with children younger than 5 years, schoolchildren, non-pregnant adults and pregnant women being exposed to mosquito bites on a 1:3:4:8 ratio.

Figure I. Contribution of infections in pregnant women to local malaria epidemiology



There are other factors that might exacerbate the hypothesised increased infectiousness of pregnant women compared to non-pregnant women and men of similar age. Pregnant women have been reported to be more attractive to mosquitoes over both short [33] and long distances [34]. Additionally, behavioural changes might also increase availability to mosquitoes: pregnant women leave their bed at night more often than non-pregnant women [34] and may thus be unprotected by their net for longer periods of time. In our calculations in **Box 1**, we assumed that attractiveness to mosquitoes increases monotonically with age, with children younger than 5 years, schoolchildren and adults (aged 15 years or more) being exposed to mosquito bites on a 1:3:4 ratio [10], and that pregnant women are twice as attractive to *Anopheles* mosquitoes compared to non-pregnant adults. Under these assumptions, and after incorporating data on the average proportion of infected mosquitoes in positive feeding experiments, the figure in **Box 1** shows estimates of the contribution of pregnant women to mosquito infections in endemic areas, adjusted for this potential increase in mosquito exposure during pregnancy. Up to 10% of mosquito infections might originate from pregnancy-associated malaria parasites.

In areas of lower transmission intensity, particularly those approaching elimination of *falciparum* malaria, women will be unlikely to be infected in each pregnancy, and the first malaria infection while pregnant will occur more frequently in multigravidae. Interestingly, recent data from southern Mozambique suggest that, when malaria transmission transitions to lower levels, the total number of infected pregnant women decreases while their parasite densities during infections increase [35]: the prevalence of PCR-confirmed maternal *P. falciparum* infections dropped from 33% in 2003-2004 to 6% in 2012, while peripheral and placental parasite densities in pregnant women were approximately ten times higher in the latter period. Whether this increase in asexual stage parasitaemia was accompanied by a similar increase in peripheral gametocytaemia is not known. However, these observations suggest that onward transmission from pregnant women remains relevant in low endemic settings.

Reducing transmission from pregnant women

Malaria transmission from pregnant women could be reduced by 1) ensuring that infections in pregnant women are promptly and effectively treated and 2) reducing the

probability that mosquitoes feed on infectious pregnant women. In many malaria endemic countries, both intermittent preventive treatment (IPTp) and insecticide treated nets (ITN) are targeted to pregnant women to reduce the burden of disease in this group and in their offspring [36]. IPTp with sulfadoxine-pyrimethamine (SP) is currently recommended as a single dose at each scheduled antenatal care visit after the first trimester, provided that these visits occur at least one month apart (www.who.int/malaria/iptp_sp_updated_policy_recommendation_en_102012.pdf). IPTp-SP clears existing infections and provides prophylaxis [37], reducing prevalence of infection [38, 39] both in the placenta and in the peripheral blood, and as a consequence is expected to limit infectiousness by reducing the duration of parasite, including gametocyte, circulation. The direct effects of SP on parasite transmission due to post-treatment gametocytaemia, on the other hand, are less clear. Whilst SP has been shown in *in vitro* studies and in field studies recruiting non-pregnant adults and children to increase gametocytaemia post-treatment [40-42], mosquito feeding assays performed on individuals treated with SP suggest that the infectiousness of these gametocytes is comparatively lower [43] but persists for at least two weeks after treatment [44]. The continuing spread of SP resistance [45] could also influence post-IPTp infectivity. Pooled analysis of clinical data generated in different sites in Sub-Saharan Africa with a wide range of SP resistance prevalence showed that where malaria parasites routinely carried mutations linked to SP resistance up to 40% of pregnant women receiving SP carried falciparum parasites on day 42 after treatment administration [46]. Whether SP resistant parasites persist, or reinfect, through multiple IPTp doses will determine the total duration of the infectious period for pregnant women living in these areas. Encouragingly, in the same multisite study, IPTp-SP was associated with beneficial effects on birth weight and risk of maternal anaemia, including in areas with high SP resistance. Taken together, these studies suggest that where SP resistance prevails, although IPTp with SP is still an efficacious strategy to prevent falciparum-related morbidity, it might not prevent malaria transmission from pregnant women.

An alternative to SP, dihydroartemisinin-piperaquine (DHA-PPQ) is effective and safe for treating malaria in pregnancy [47, 48] and will now be evaluated to assess its safety and effectiveness within IPTp regimens. Although residual falciparum transmission to mosquitoes has been observed after the administration of DHA-PPQ [49, 50], this

regimen shortens the overall infectious period through effective clearance of malaria parasites, including of immature gametocytes, and could reduce transmission from pregnant women. This effect would be maximal in the second and third trimesters of pregnancy when IPTp is recommended and infectiousness and mosquito attractiveness would be greatest. Of note, twenty-year prospective observational data collected in antenatal clinics in the Thai-Myanmar border [51] suggest that treatment of falciparum malaria in the first trimester of pregnancy with artemisinin derivatives is not associated with an increased risk of miscarriage or major malformation diagnosed by clinical examination compared to treatment with quinine. These findings and new evidence reported in a recent WHO recommendation (www.who.int/malaria/publications/atoz/istp-and-act-in-pregnancy.pdf) indicate that artemisinin-based combination therapies (ACTs) might be a safe alternative for malaria treatment also during the first trimester of pregnancy.

In addition to IPTp, ITNs, the other key strategy for the prevention of malaria in pregnancy [52], also have the benefit of decreasing the probability of women with malaria in pregnancy transmitting their infections to mosquitoes. In **Figure 1B**, we illustrate how these preventive measures can influence malaria transmission potential of pregnant women. Despite the benefits associated with them, the proportion of pregnant women in Sub-Saharan Africa at risk of malaria infection who receive IPTp or sleep under ITN remains highly variable and is particularly low in primigravidae: recent model-based estimates suggest that in 2015 only 35% of women whose pregnancy could result in malaria-attributable low birth weight used bed nets and 21.6% of all pregnant women at risk of malaria infection received at least two doses of IPTp-SP (Walker et al, in press). IPTp and ITN are therefore unlikely to prevent pregnancy-associated malaria parasites from sustaining transmission during mass chemotherapy campaigns. Although IPTp is a form of demographically-targeted MDA, it is likely to be less effective at reducing transmission compared to community-wide MDA because IPTp is asynchronous (it does not clear all infections over a short time-window), because SP does not clear gametocytes, and because the parasitological efficacy (ability to clear blood-stage infection) of SP is low in areas of SP resistance whilst it may still be clinically effective (i.e. effectively reduce clinical consequences for mother and offspring).

Community-based diagnosis of infection in potentially pregnant women during MDA could boost effectiveness and improve maternal health

Given the factors outlined above, elimination programmes should reconsider the exclusion of pregnant and potentially (based on the timing or lack of knowledge of their last menstrual period) pregnant women, especially in areas where IPTp is not being implemented or not being effective due to poor implementation or parasite resistance. Indeed, the high sensitivity of RDTs for placental infection in women with few previous infections in pregnancy (**Figure 1A**) suggests that providing RDT-based diagnosis and treatment with effective antimalarials as part of the MDA campaign could substantially reduce the number of high density placental infections present within the population. As transmission approaches elimination levels, parasite burden during pregnancy-associated malaria infections increases and the proportion of these infections with parasitaemia above the threshold for RDT positivity might also increase, suggesting that this approach could be used throughout elimination campaigns. Where preventive treatment is absent or infrequent, the community-based diagnosis of malaria infections in pregnancy would result in both improvements in the effectiveness of the MDA in reducing transmission and preventing the likely adverse pregnancy outcomes associated with malaria infection. In areas where IPTp has been successfully implemented, if ACTs are found to be an acceptable alternative to SP as preventive therapy during pregnancy, MDA campaigns could work with antenatal care clinics to identify women who have not been regularly treated and need to receive the MDA treatment. For women of reproductive age who do not have pregnancy diagnosis at the time of an MDA round, a pregnancy test would need to be performed: those women with negative pregnancy test result would immediately receive MDA treatment; those with positive pregnancy test result would be tested for malaria infection. Only pregnant women with negative malaria RDT result would not receive MDA treatment. In this approach, pregnancy-testing during MDA campaigns might also promote early identification of pregnant women, who need or will eventually need to receive IPTp doses. Pregnancy testing during mass treatment campaigns that use primaquine as part of the chemotherapy would be particularly important as this drug is contraindicated in pregnant women [53]. On the other hand, screening for pregnancy will increase direct costs of MDA campaigns and might discourage participation by women who do not want to be tested. National guidelines for mass chemotherapy

should be followed and, in areas where pregnant women are included in MDA campaigns, enhanced pharmacovigilance will increase the probability of detecting rare teratogenic effects.

Concluding Remarks

Annually, more than 85 million pregnancies occur in areas with falciparum transmission, 30 million only in Africa [54]. Previous efforts to reduce malaria transmission levels using community-based chemotherapy approaches often excluded pregnant women, despite the lack of data on their transmission potential. The high parasite levels observed during pregnancy suggest that these ‘non-targeted’ individuals might harbour infections with higher densities of transmissible gametocytes compared to infections in non-pregnant adults, and this could result in high infectivity. Their attractiveness to mosquitoes also means that pregnant women are frequently sampled by malaria vectors and their gametocytes have more opportunities to be ingested by mosquitoes. Together, these observations suggest that pregnant women might become an important source of mosquito infections after MDA rounds with high coverage. Accumulating evidence from clinical and epidemiological studies indicate that the highly effective ACTs could be used to clear infections of pregnant women, who would be screened for malaria with RDTs during MDA campaigns. Studies assessing the intensity and duration of falciparum infectiousness during different trimesters of pregnancy (see *Outstanding Questions* box) are necessary to develop accurate mathematical models of the contribution of pregnant women to local transmission and will be important to support the planning of these interventions.

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Competing interests

The authors have declared that no competing interests exist.

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Appendix C, Molecular quantification of gametocytes

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Student	Bronner Pamplona Augusto Gonçalves
Principal Supervisor	Chris Drakeley
Thesis Title	The human infectious reservoir of falciparum malaria

If the Research Paper has previously been published please complete Section B, if not please move to Section C

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For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper	I was involved in field activities, performed statistical analyses and wrote the first draft of the manuscript with Helmi Pett.
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Student Signature: Bronner P. A. G. Date: 04/09/2017

Supervisor Signature: Chris Drakeley Date: 04/09/2017

Title: Comparison of molecular quantification of *Plasmodium falciparum* gametocytes by *Pfs25* qRT-PCR and QT-NASBA in relation to mosquito infectivity

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Abstract

Background

Quantifying gametocyte densities in natural malaria infections is important to estimate malaria transmission potential. Two molecular methods (*Pfs25* mRNA quantitative reverse transcriptase PCR (qRT-PCR) and *Pfs25* mRNA quantitative nucleic acid sequence based amplification (QT-NASBA)) are commonly used to determine gametocyte densities in clinical and epidemiological studies and allow gametocyte detection at densities below the microscopic threshold for detection. Here, reproducibility of these measurements and the association between estimated gametocyte densities and mosquito infection rates were compared.

Methods

To quantify intra- and inter-assay variation of QT-NASBA and qRT-PCR, a series of experiments was performed using culture-derived mature *Plasmodium falciparum* gametocytes from three different parasite isolates (NF54, NF135, NF166). *Pfs25* mRNA levels were also determined in samples from clinical trials in Mali and Burkina Faso using both methods. Agreement between the two methods and association with mosquito infection rates in membrane feeding assays were assessed.

Results

Intra- and inter-assay variability was larger in QT-NASBA compared to qRT-PCR, particularly at low gametocyte densities (<1 gametocyte per μL). Logistic models, including log-transformed gametocytaemia estimated by QT-NASBA, explained variability in mosquito feeding experiment results as well as log-transformed gametocytaemia by qRT-PCR (marginal R^2 0.28 and 0.22, respectively); densities determined by both methods strongly correlated with mosquito infection rates (Spearman's rank correlation coefficient, 0.59 for qRT-PCR and 0.64 for QT-NASBA ($P < 0.001$ for both)). Gametocyte densities estimated by qRT-PCR were higher than levels estimated by QT-NASBA or light microscopy at high densities (>100 gametocyte per μL). Samples collected in one of the two transmission studies had extremely low gametocyte densities by both molecular methods, which is suggestive of RNA degradation due to an unknown number of freeze-thaw cycles and illustrates the reliance of molecular gametocyte diagnostics on a reliable cold-chain.

Conclusions

The experiments indicate that both qRT-PCR and QT-NASBA are of value for quantifying mature gametocytes in samples collected in field studies. For both assays, estimated gametocyte densities correlated well with mosquito infection rates. QT-NASBA is less reproducible than qRT-PCR, particularly for low gametocyte densities.

Background

The transmission of malaria from humans to mosquitoes requires the presence of mature sexual stage parasites (gametocytes) in the peripheral blood. Upon ingestion by *Anopheles* mosquitoes and following sporogonic development, these gametocytes can render mosquitoes infectious to humans. Quantifying the density of these gametocytes in the peripheral blood is a fundamental part of estimating the infectiousness of individuals with malaria infections. For over a century, light microscopy was the only method available to quantify malaria parasites, including gametocytes. The development of sensitive molecular methods has uncovered some of the limitations of microscopy. In endemic areas, a substantial proportion of infected individuals carry parasites at levels below the microscopic threshold of detection [1-4], and many of these individuals also have low, sub-microscopic, densities of gametocytes [5, 6]. Together with early observations that individuals with no gametocytes detected by microscopy could be infectious to *Anopheles* mosquitoes [7], these studies suggest that the use of more sensitive methods is necessary to characterize the infectious reservoir of malaria [8, 9].

The most widely used target for molecular gametocyte detection and quantification is *Pfs25* mRNA [9], which is gametocyte-specific and highly conserved among different parasite isolates [6]. Although expression of *Pfs25* mRNA is upregulated in female gametocytes [10], the female-biased gametocyte sex ratio and the high abundance of *Pfs25* mRNA relative to the male specific marker *Pfs230p* make it an attractive target for sensitive gametocyte detection and quantification [11].

Various molecular techniques have been used for gametocyte detection and quantification. For *Pfs25* mRNA there are two commonly used protocols, one based on quantitative reverse transcriptase PCR (qRT-PCR) and another based on quantitative nucleic acid sequence based amplification (QT-NASBA) [6, 12]. Although previous studies on other malaria-specific targets suggest that the accuracy, precision and operational attractiveness may differ between assays [13], QT-NASBA and qRT-PCR, as currently routinely used, have never been directly compared for *Plasmodium falciparum* gametocyte quantification. Additionally, differences in *Pfs25*

transcript levels between malaria parasite strains have been hypothesized but never directly examined [14].

Here, *Pfs25* mRNA QT-NASBA and qRT-PCR were compared for intra- and inter-assay variation using gametocytes from three different isolates [6, 12]. The association between gametocyte densities estimated by these two molecular methods in natural malaria infections and infectiousness quantified by membrane feeding assays was also determined.

Methods

Study design

In this study, both parasite culture-derived samples and samples from naturally infected individuals were used. For the estimation of intra-assay variation for both QT-NASBA and qRT-PCR, culture-derived mature *P. falciparum* gametocytes at known densities were assayed in triplicates on the same 96-well plate with the same reaction mixtures; for the inter-assay variation analysis, gametocyte dilution series were tested on separate plates and with separately prepared reaction mixtures. Samples collected from naturally infected individuals living in malaria-endemic regions were used to assess the correlation between gametocyte levels measured by microscopy, qRT-PCR and QT-NASBA and human infectiousness to mosquitoes, determined by mosquito feeding experiments.

Gametocyte culture

Plasmodium falciparum gametocytes from three laboratory parasite strains (NF54, NF135, NF166) were cultured in shaker flasks, as previously described [15-17]. Briefly, asexual blood stage parasites were reseeded at 5% haematocrit (erythrocytes provided by healthy Dutch donors with blood type A) and 0.5% parasitaemia in culture medium containing RPMI 1640 with HEPES (5.94 g/L), hypoxanthine (0.05 g/L), 10% (v/v) pooled human sera (also blood type A) obtained from malaria-naïve individuals, and 0.2% (w/v) sodium bicarbonate. Culture medium was automatically changed twice a day, and on day 4 50 mM of *N*-acetylglucosamine (NAG) was added in order to prevent the next generation of merozoites from infecting new erythrocytes. Gametocytes were allowed to mature until day 14.

Gametocyte dilution series

On day 14, mature gametocytes were harvested and Percoll purified according to the protocol described in Kariuki *et al.* [18]; 63%, instead of 65%, Percoll gradient was used. Cultures and material used for purification were continuously kept at 37°C to avoid gametocyte activation. Following Percoll purification, gametocytes were counted by a single reader using a haemocytometer and diluted in whole blood at densities of 10^3 , 10^2 , 10^1 , 10^0 , 10^{-1} , and 10^{-2} or 5×10^3 , 5×10^2 , 5×10^1 , 5×10^0 , 5×10^{-1} , and 5×10^{-2} gametocytes per μL . Fifty μL aliquots of each dilution were further diluted in 250 μL of RNeasy Protect Cell Reagent (Qiagen) when analysing standard curve triplicates and samples collected in Burkina Faso (see below) or in 450 μL of L6 buffer when analysing samples collected in Mali, and stored at -80°C until further use [19].

Samples from naturally infected individuals in clinical trials

Blood samples from patent gametocyte carriers were collected during clinical trials in Mali and Burkina Faso [20, 21]. In the study undertaken in Mali, male participants with at least 32 gametocytes per μL of blood based on gametocyte enumeration against 500 white blood cells (WBC), assuming 8000 WBCs/ μL , were recruited. The trial in Burkina Faso had as an inclusion criterion the presence of at least one microscopically detectable gametocyte at screening slides, where 100 microscopic fields were examined. In both studies, blood samples were taken at multiple time-points, before and after treatment initiation, to assess gametocyte clearance. Samples (venous blood in EDTA (Mali) or heparin (Burkina Faso) vacutainer) taken before initiation of anti-malarials were used in the current study. In Burkina Faso, 50 μL aliquots of blood were transferred into 250 μL of RNeasy Protect Cell Reagent. In Mali, 100 μL of blood were transferred into 900 μL of L6 buffer. Both sets of samples were kept at -80°C until shipment, although the samples collected in Burkina Faso may have been exposed to freeze-thaw due to power-cut of unknown duration during political unrest in November 2014. For both studies, samples were shipped in dry ice at controlled temperature (-70 to -80°C) to Radboud University Medical Centre (Nijmegen, The Netherlands), where the laboratory work was performed.

For all samples, total nucleic acids (NA) were extracted using a MagNAPure LC automatic extractor (Total Nucleic Acid Isolation Kit – High Performance, Roche

Applied Science) and eluted into 50 μL of elution buffer; while all of the 300 μL of samples collected in Burkina Faso were extracted, only half of the volume (500 μL of the 1 mL) of samples collected in Mali were used for total NA extraction.

Mosquito membrane feeding experiments

All individuals, whose samples were included in this analysis, participated in mosquito feeding experiments: a small quantity of heparinized venous blood (400-500 μL) was offered to female *Anopheles gambiae* mosquitoes (180 mosquitoes in the Mali study and 60 in the Burkina Faso study) through a membrane [20-22]. Fully fed mosquitoes at the end of each experiment were selected, kept for one week on glucose at 27-29°C to allow for parasite development, and dissected. In both studies, light microscopy was used to detect oocysts on the midgut wall, and their presence was confirmed by a second microscopist. The proportion of mosquitoes with at least one oocyst was used as outcome measure.

***Pfs25* mRNA qRT-PCR**

The use of reverse transcriptase PCR (RT-PCR) for the detection of *Pfs25* mRNA was first described by Babiker *et al.* [23]; recently it was further developed into a qRT-PCR [6]. Here, a protocol described by Schneider *et al.* was used [11] with primers designed by Wampfler *et al.* [6]. Briefly, total NA extraction was followed by RQ1 DNaseI digest (Promega) and cDNA synthesis (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems). The DNase treatment and the RT-step were done once for each sample; the qPCR step was done in triplicate for samples included in the intra-assay variation analysis. Success of the DNase treatment was tested by performing qPCR, with the same primers, on two to five randomly selected samples of each plate (in total 35 samples) before proceeding to the RT-step. No residual genomic DNA was detected in these assays, which indicates successful DNase treatment. PCR conditions were according to manufacturer's instructions, described as standard cycling programme of GoTaq® qPCR Master Mix (Promega). qRT-PCR cycle of threshold (Ct) values (the cycle at which the fluorescence signal crosses a pre-determined threshold during the exponential phase of the amplification) were obtained using CFX96™ Real-Time PCR Detection System (BIO-RAD). For samples from naturally infected individuals, Ct values were converted into gametocyte density using plate-specific standard curve. Samples with estimated densities below 0.02

gametocytes per μL or 1 gametocyte per 50 μL of sample were considered negative. At the highest density of the gametocyte dilution series, cDNA corresponding to 363.6 gametocytes was estimated to have been included in qRT-PCR reactions (Additional file 1).

***Pfs25* mRNA QT-NASBA**

QT-NASBA was performed as in Schneider *et al.* [12]. KCl concentrations in experiments performed for this study varied from 50 to 60 mM, and differed from the concentration used in the original protocol (80 mM) [12]. For samples from the Burkina Faso study, the extracted total NA used for qRT-PCR was also used for QT-NASBA (before DNase treatment and cDNA production). For samples collected in the Mali trial, different aliquots of the same sample were extracted (total NA) and used for the different molecular assays; the QT-NASBA aliquot did not undergo DNase treatment and cDNA production. QT-NASBA results are expressed as time to positivity (TTP), as in this method amplification happens at a constant temperature, not in cycles. Standardized manual thresholds to remove background noise are set at the begin value of 3 min and the end value of 15 min. As with qRT-PCR, gametocyte densities were assigned based on plate-specific gametocyte dilution series, which was diluted in whole blood before extraction of total NA. For samples from infected individuals, estimated gametocyte densities below 0.02 gametocytes per μL were considered to be negative. At the highest density of the gametocyte dilution curve, total NA corresponding to 2,500 gametocytes was estimated to have been included in QT-NASBA reactions (Additional file 1).

Statistical analysis

Analyses were conducted using STATA version 12 (Stata Corporation, College Station, TX, USA) and R (R Foundation for Statistical Computing, Vienna, Austria).

Intra-assay variation was assessed by estimating coefficients of variation (CV, expressed in %) (i.e., standard deviation divided by mean, and multiplied by 100) for each level of gametocytaemia tested in triplicate, in culture-specific gametocyte dilution series. Gametocyte dilution series were also used to quantify inter-assay variation: CV was estimated for each gametocyte density for all series tested in different reaction plates. To analyse the agreement between qRT-PCR- and QT-

NASBA-defined gametocyte levels in samples from infected individuals in the Mali study, a Bland-Altman plot was constructed, whereby differences between densities assigned by these two methods are presented by the average assigned level. Generalized linear mixed models, with study participant as random effect, were fit to assess the association between mosquito infection status in feeding assays and gametocyte densities (fixed effect) assigned by these two molecular methods; for each quantification method, Akaike information criterion was used to select gametocyte density scale (linear or logarithmic) in the models. Marginal R^2 , variance explained by fixed effects, was estimated using the 'r.squaredGLMM' function in MuMIn package in R [24]

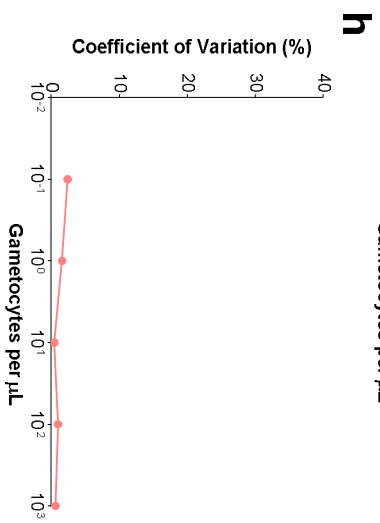
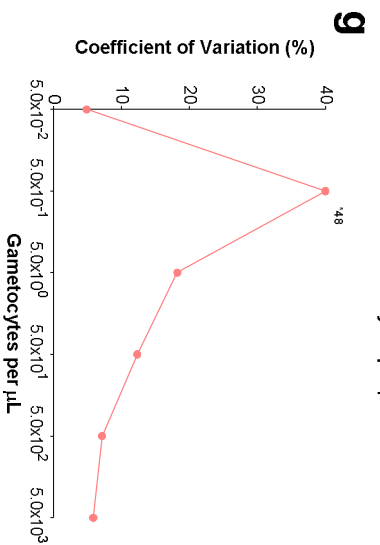
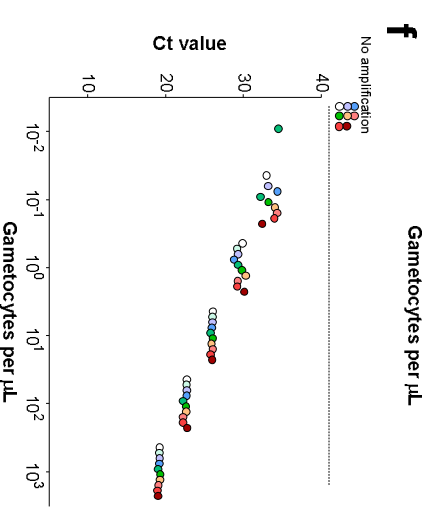
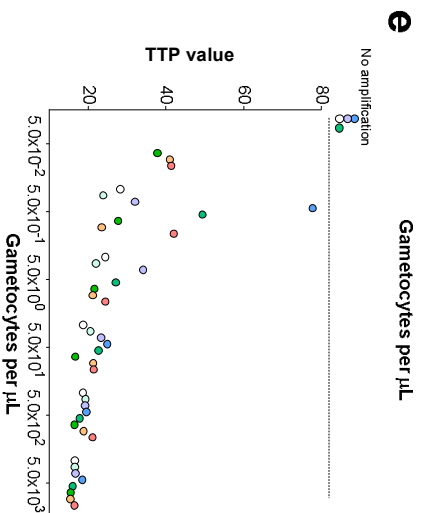
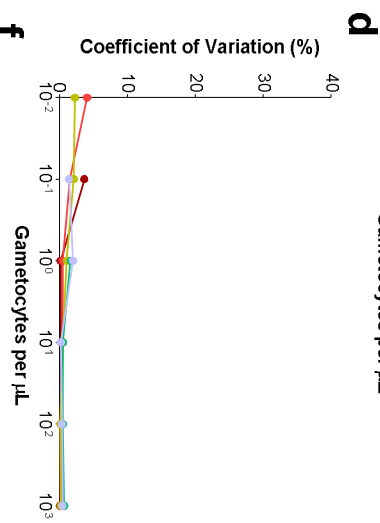
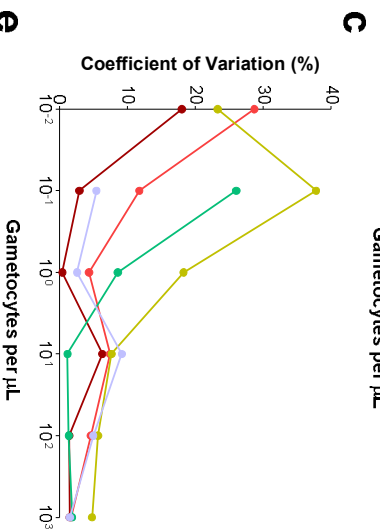
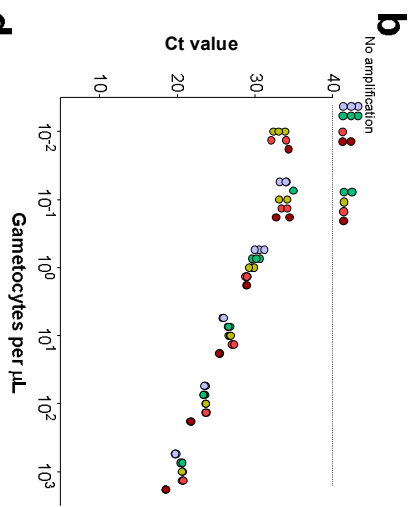
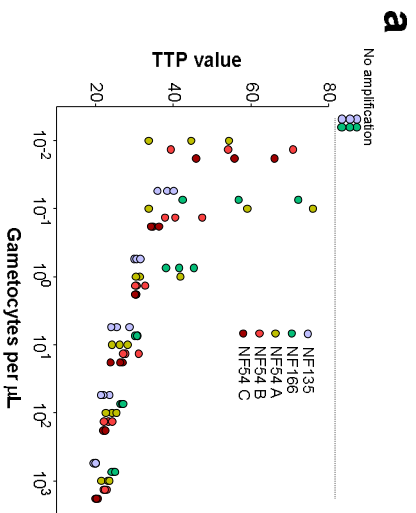
Results

Intra-assay variation

Plasmodium falciparum gametocytes of the NF54, NF135 and NF166 strains were used to prepare dilution series with 10^3 , 10^2 , 10^1 , 10^0 , 10^{-1} , and 10^{-2} gametocytes per μL . Additionally, for the NF54 strain, samples originating from three different culture flasks were tested. For each gametocyte density of individual dilution series, *Pfs25* mRNA expression was analysed in triplicate, using both qRT-PCR and QT-NASBA.

In Fig. 1 (panels A and B), TTP and Ct values for QT-NASBA and qRT-PCR, respectively, are presented. For both assays, *Pfs25* mRNA was not detected at the lowest gametocyte densities (0.01 gametocytes per μL) of the NF135 and NF166 dilution series. Although results from dilution series appeared comparable between all three *P. falciparum* strains, the failure to detect the lowest gametocyte concentration for NF135 and NF166 might indicate lower *Pfs25* transcript levels compared to the NF54 strain that only affects detection and quantification at extremely low densities. Additionally, in 5/15 0.10-gametocytes-per- μL samples, *Pfs25* mRNA was not detected by qRT-PCR. In samples where amplified *Pfs25* transcript was detected CV were inversely related to gametocyte levels and much lower for qRT-PCR compared to QT-NASBA at all gametocyte densities.

Figure 1. Intra (A-D) and inter (E-H) assay variation of QT-NASBA and qRT-PCR. In A-D, different colours represent different strains and cultures used for intra-assay variation assessment. In E-H, different experiments used in the inter-assay variation assessment are represented by different colours. Time to positivity (TTP) values for samples tested by QT-NASBA are presented in A and E and coefficients of variation (CVs), in C and G. Cycle of threshold (Ct) values for samples tested by qRT-PCR are presented in B and F; CVs are presented in D and H. In A, B, E and F, circles outside the y-axis range correspond to samples where *Pfs25* mRNA was not detected. Of note, for experiments included in inter-assay variation analysis, different densities were used in gametocyte dilution series for QT-NASBA and qRT-PCR.



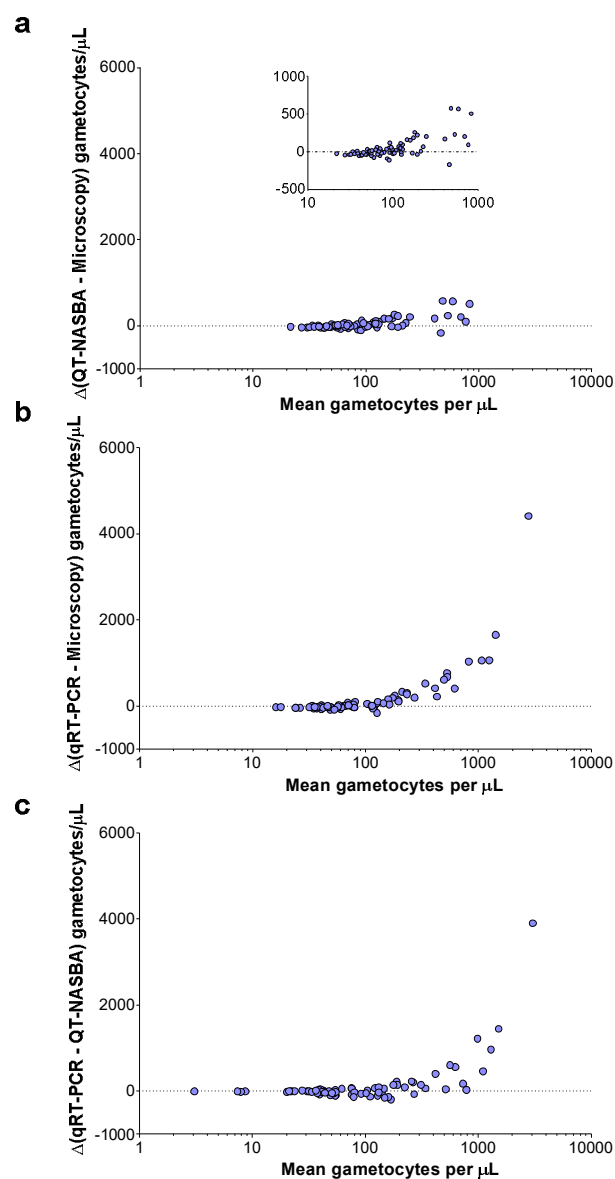
Inter-assay variation

Seven (including the following densities 5×10^3 , 5×10^2 , 5×10^1 , 5×10^0 , 5×10^{-1} , and 5×10^{-2} gametocytes per μL) and ten (including the densities 10^3 , 10^2 , 10^1 , 10^0 , 10^{-1} , and 10^{-2} gametocytes per μL) gametocyte dilution series, each tested on a different plate, were used to assess QT-NASBA and qRT-PCR inter-assay variation, respectively. Only NF54 strain parasite cultures were used in these experiments. Samples with the lowest density in each series were often negative for *Pfs25* mRNA. Despite the higher densities used for the QT-NASBA *versus* qRT-PCR dilution series, a similar pattern to the intra-assay variation experiments was observed (Fig. 1, panels G and H): CVs were considerably higher for QT-NASBA compared to qRT-PCR and decreased with increasing gametocyte densities.

Gametocyte densities in naturally infected individuals

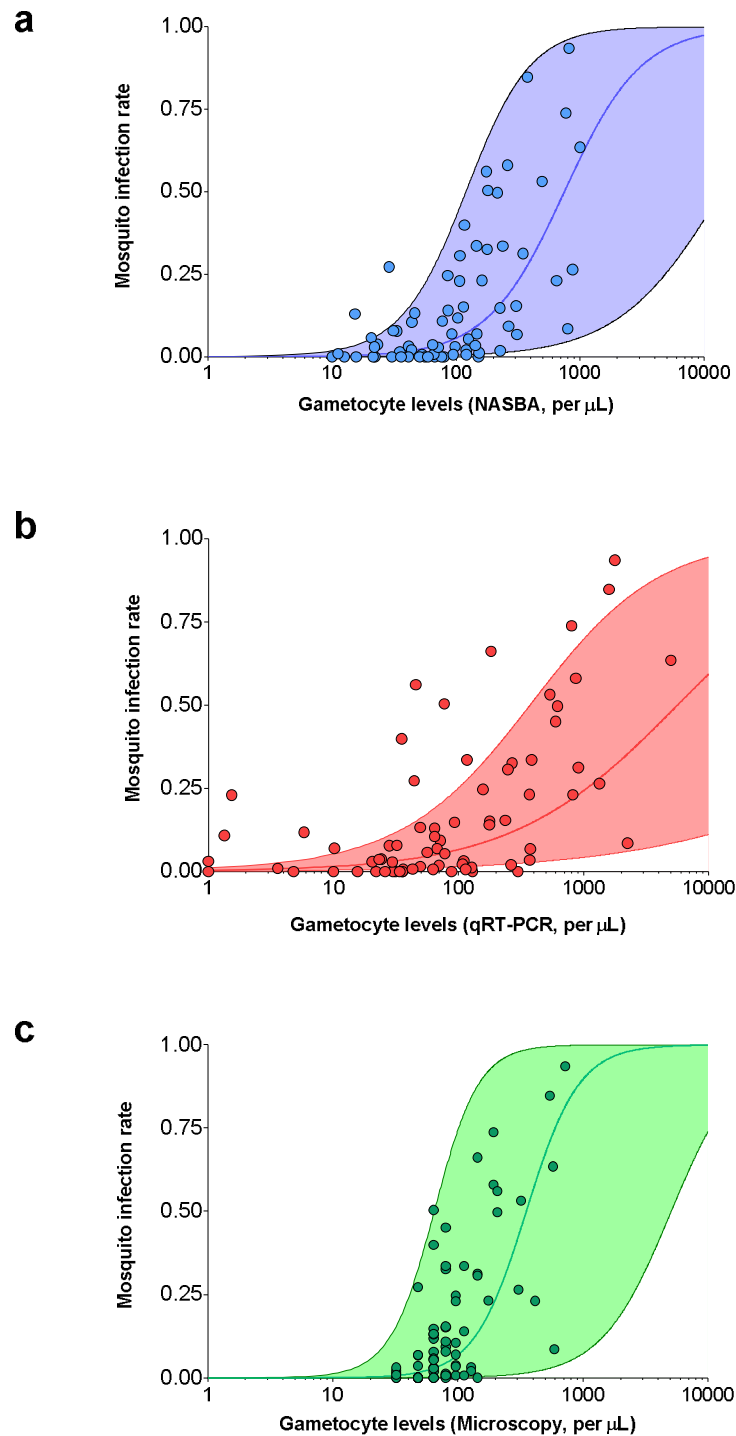
Blood samples collected prior to treatment from gametocytaemic individuals participating in mosquito feeding experiments in Mali were analysed by microscopy, qRT-PCR and QT-NASBA. For the two molecular assays, gametocyte densities were estimated based on dilution series of culture-derived *P. falciparum* gametocytes of the NF54 strain tested in the same plate, as routinely done [5, 12]. As a consequence of enrolment criteria, all individuals (N=73) had microscopically detectable gametocytes at the time of the feeding assay (median (IQR) 80 (64-112) gametocytes per μL), and all their samples were assigned gametocyte levels above the threshold of positivity for both molecular assays (minima gametocyte levels were 0.04 and 6.1 gametocytes/ μL for qRT-PCR and QT-NASBA, respectively). Median (IQR) gametocytaemias estimated by QT-NASBA and qRT-PCR were similar: 94.8 (41.6-180.7) and 69.8 (29.8-263.0) gametocytes per μL , respectively. The agreement between microscopy and QT-NASBA, between microscopy and qRT-PCR and between the two molecular methods was assessed by Bland-Altman plots (Fig. 2). At high gametocyte concentrations (>100 gametocytes per μL), gametocyte densities estimated by qRT-PCR were higher than densities estimated by microscopy or QT-NASBA.

Figure 2. Bland-Altman plots to assess agreement between light microscopy, QT-NASBA and qRT-PCR levels in samples from naturally infected individuals. In the y-axes, differences between gametocyte levels estimated by two different methods are presented: (A) QT-NASBA levels minus microscopy-defined densities, (B) qRT-PCR minus microscopy levels, (C) qRT-PCR levels minus levels estimated by QT-NASBA. The x-axes present the average of the densities estimated by the two methods included in the calculation of the respective y-axes. In panel A, the differences (y-axis) are limited to a narrower range of values compared to panels B and C, and the inset plot presents the same data using different y-axis limits.



In the same study in Mali, 73 membrane-feeding assays were performed. The median (IQR) number of mosquitoes fed and dissected was 176 (174-177) and 142 (130-152), respectively. Some 58/73 (79.4%) individuals infected at least one mosquito, and the median (IQR) mosquito infection rate (proportion of infected mosquitoes, i.e., proportion of mosquitoes with \geq one oocyst) was 12.4% (3.4-32.6). Gametocyte levels were lower in non-infectious *versus* infectious individuals (Additional file 2), and correlated with mosquito infection rates (Spearman's rank correlation coefficient 0.59, 0.62 and 0.64 ($P < 0.001$ for all assays) for qRT-PCR, microscopy and QT-NASBA, respectively). Statistical models including log-transformed gametocyte levels estimated by QT-NASBA or microscopy explained a slightly higher proportion (marginal $R^2 = 0.28$ and 0.26 , respectively) of infectiousness variability compared to models including log-transformed densities by qRT-PCR (marginal $R^2 = 0.22$) (Fig. 3).

Figure 3. Mosquito infection rates (y-axes), presented as proportions, by gametocyte counts (x-axes) in samples collected in a clinical study in Mali. Gametocyte densities were estimated by QT-NASBA (A), qRT-PCR (B) and light microscopy (C). Fitted curves and 95% confidence intervals were estimated using glmmadmb package in R.



Lower-than-expected gametocyte densities in molecular assays

Venous samples collected from 77/79 children participating in mosquito-feeding experiments in Burkina Faso were also analysed. Of these 77 children, 27 did not carry microscopically detectable gametocyte densities on the day of their membrane feeding assay, despite having gametocytes detected by microscopy during the screening phase of the study (one to three days earlier). Median (IQR) gametocyte density determined by microscopy was 12 (0-24) gametocytes per μL . Twenty of 77 did not have *Pfs25* mRNA detected by qRT-PCR, and four had assigned gametocyte levels between 0 and 0.02 gametocytes per μL , and were considered gametocyte negative. However, ten of these 24 children with no gametocytes detected by qRT-PCR had gametocytes detected by microscopy. Gametocyte densities estimated by QT-NASBA were also very low for most samples: 55 children, including 31 with microscopically detectable gametocytes, had assigned levels below 0.02 gametocytes per μL (and were thus classified as gametocyte negative) and for one child of 76 *Pfs25* mRNA was not detected. All 23 samples with no *Pfs25* mRNA amplification or with densities below 0.02 gametocytes per μL by qRT-PCR and that were also tested by QT-NASBA had assigned densities below 0.02 gametocytes per μL by this method. Considering that all these children had microscopically detectable gametocytes one to three days before sample collection, it is likely that RNA material was of insufficient quality. It is highly plausible that freeze-thaws with unknown duration or frequency have affected RNA integrity of this set of samples, all kept in the same box throughout the storage period and shipment. The infectivity of study participants in the clinical trial in Burkina Faso was lower than the infectivity of individuals enrolled in the Malian study: 29/77 (37.6%) children infected at least one mosquito in feeding experiments. Although differences in the prevalence of host factors that influence infectivity, such as transmission-blocking immunity [25] or haemoglobinopathies [26], could have contributed to this observation, this disparity in infectiousness prevalence is probably due to real differences in gametocyte levels as suggested by microscopy-based quantification and differences in the number of dissected mosquitoes (median number of mosquitoes dissected per assay, 142 and 45 in Mali and Burkina Faso, respectively). The facts that five of 29 and 17 of 29 individuals who infected mosquitoes did not have *Pfs25* mRNA detected by qRT-PCR or had very low assigned levels (<0.02 gametocytes per μL) by QT-NASBA, respectively, and that the gametocyte density-

mosquito infection rate plot is shifted to the left compared to the corresponding graph for the Malian study (Additional file 3) support the freeze-thaw explanation.

Discussion

In this study, *Pfs25* mRNA QT-NASBA and qRT-PCR were evaluated using culture-derived gametocyte samples and samples from naturally infected individuals. In *in vitro* experiments, both intra- and inter-assay variations were lower for qRT-PCR compared to QT-NASBA. When analysing samples from naturally infected individuals, gametocyte densities estimated by qRT-PCR were higher than densities estimated by QT-NASBA and microscopy in individuals with high gametocyte levels. Gametocyte densities estimated by the two molecular assays, as well as by microscopy, correlated well with mosquito infection rates. Overall, the findings indicate that both assays are useful for quantifying sexual stage parasite densities in samples from naturally infected individuals, but qRT-PCR showed better reproducibility.

Determining the variability of quantitative assays is important for the interpretation of results. The experiments here suggest that qRT-PCR has lower intra- and inter-assay variation compared to QT-NASBA. The higher precision of qRT-PCR is evident for the entire range of gametocyte densities used in the dilution series, despite involving extra reaction steps, such as DNase treatment and cDNA production, which are known to be a source of variation [27]. These two steps were performed once for each dilution series density, for the three different NF54 cultures and the NF135 and the NF166 cultures. There was considerably less between-strain and -culture variation for similar densities in qRT-PCR *versus* QT-NASBA. In QT-NASBA reactions, from the time of primer depletion, amplification will mostly depend on the initial number of transcripts, resulting in a linear increase of amplicons. This exact moment when amplification starts may differ between samples depending on when and how the enzyme is added into the reaction wells, which adds a level of uncertainty to this methodology [28]. The range of TTP values in dilution series is also sensitive to the KCl concentration in the mix, which requires regular optimization with new targets, batches of primers or molecular beacons [29].

Despite qRT-PCR being a more precise method for gametocyte quantification, QT-NASBA might detect a larger number of very low gametocyte densities (<0.1 gametocytes per μL). QT-NASBA was originally developed for highly sensitive qualitative detection. The apparent higher sensitivity of QT-NASBA compared to qRT-PCR may be explained by methodological differences: for QT-NASBA it is not necessary to dilute samples, in order to complete DNase treatment and cDNA production (Additional file 1) and the equivalent of a larger volume of the original sample is therefore added into the final reaction in QT-NASBA compared to qRT-PCR. This additional dilution in the qRT-PCR protocol could be avoided by using column-based RNA extraction with on-column DNase digestion [6]. Also, while qRT-PCR, in the protocol used here, carries on for a set amount of 40 cycles, the QT-NASBA reaction is continuous at a stable temperature for the duration of 90 minutes. This might allow for more sensitive amplification of low numbers of transcripts, but could also lead to the detection of single stranded DNA, in cases in which genomic DNA has been degraded and exists in the sample in a single stranded form. A stringent cut-off density below which samples are considered negative must therefore be applied in QT-NASBA, as with all other molecular assays.

Whether qRT-PCR overestimates true gametocyte levels or QT-NASBA and microscopy underestimate densities is not clear. When qRT-PCR is compared with the other methods, high mean densities correspond to the qRT-PCR measurement being larger than the other measurements. There are two possible reasons for observing this pattern: 1) qRT-PCR overestimates high gametocyte concentrations; or, 2) qRT-PCR measurements have greater variability [30]. Since the *in vitro* experiments suggest that qRT-PCR has lower variability than QT-NASBA, it is likely that qRT-PCR has an upward bias at high gametocyte concentrations (>100 gametocytes per μL). The overestimation is unlikely to be caused by residual DNA in the RNA samples used in the qRT-PCR analysis, as the success of the DNase treatment was tested for two to five samples per plate, with no amplification detected.

The value of molecular methods in detecting epidemiologically relevant malaria infections is becoming increasingly well established. A recent meta-analysis comparing PCR, microscopy and rapid diagnostic test (RDT) sensitivities showed that on average RDTs detect less than half of PCR-positive *P. falciparum* infections, and

microscopy detects slightly less infections than RDTs [8]. The density of these sub-microscopic infections and their concurrent gametocyte levels are of relevance in estimating the contribution to malaria transmission. For *P. falciparum* parasite density (including asexual parasites and gametocytes), RNA-based QT-NASBA was previously compared to DNA-based qPCR for the quantification of *P. falciparum* 18S RNA and DNA, respectively [13]. Another recent study, this time on *Leishmania* parasites, compared 18S rRNA-based quantification by qRT-PCR and QT-NASBA to 18S rRNA gene DNA-based detection with qPCR [31]. In the first study, QT-NASBA was found to be more convenient and equally applicable for quantification purposes as real-time PCR, with strong correlation with quantification by microscopy and similar inter-assay variation to both methods [13]. In the second study, intra- and inter-assay CVs were deemed equal for all three molecular methods and qRT-PCR was preferred over the other two methods for convenience reasons [31]. One explanation for these conflicting conclusions is the method used for detection of the amplified target in QT-NASBA: in the study comparing *Leishmania* quantification, the electrochemiluminescence (ECL) read-out added to the general workload of the assay. Additionally, co-amplification with a known amount of quantitative (Q)-RNA, as was originally done for the quantification of the different gametocyte stages as well as the *Leishmania* parasites, added an extra factor to the analysis of results [12, 31]. The direct comparison to a standard curve with microscopically determined density of parasites, in addition to the use of molecular beacons, simplified the quantification process of QT-NASBA results [13]. As mentioned earlier in the context of assay sensitivity, also convenience-wise, an important advantage of QT-NASBA is that it can be performed directly on extracted NA.

Several factors, such as the type of anticoagulant used in sample collection and the storage conditions, might influence the outcome of molecular assays on samples [9, 32]. The analysis of samples from Burkina Faso provides indirect evidence for this: the extremely low assigned gametocyte levels, including in samples collected from children with microscopically detectable gametocytes, suggest that these samples might have gone through more than one freeze-thaw cycle. In these situations, sample quality may be compromised: mRNA may be degraded and, although abundant transcripts, such as *Pfs25* mRNA, might still be detectable, quantification becomes less reliable [33]. Quantification of constitutively expressed human RNA targets and

comparison of transcript levels among samples is one method that could have confirmed whether RNA degradation occurred and is recommended for future studies where there is uncertainty about sample integrity.

Ultimately, the goal in quantifying gametocytes is to indirectly estimate human infectivity, as mosquito-feeding assays are logistically complex and only a handful of research institutes currently have the infrastructure to perform these experiments in sub-Saharan Africa. In this study, it was observed that QT-NASBA- and microscopy-defined densities explain a slightly higher proportion of the variation in infectivity compared to gametocyte levels assigned by qRT-PCR but this difference should be interpreted with caution. The statistical method used here to evaluate this relationship was recently employed to assess the association between viraemia and mosquito infection risk in feeding assays involving dengue-infected individuals [34]. While the sigmoidal curves estimated by these models seem to fit mosquito infection data well for QT-NASBA and microscopy, it is possible that qRT-PCR-defined high densities were overestimated (Fig. 2B) and that could possibly explain the poorer fit of the model for that assay. These comparisons are based on a statistical model. More complex models [35], allowing for more flexible sigmoidal curves, would be required to formally assess the shape of the association between gametocyte density and mosquito infection rates. The quantification of other parasite (e.g., sex ratio) or host factors (e.g., transmission-blocking immune responses and haematological factors) that might influence infectivity is likely to improve the predictive value of these models [36]. Of note, the enrolment criterion of multiple gametocytes being observed by microscopy (two or more gametocytes per 500 WBC) makes it difficult to directly extrapolate these results to sub-microscopic infections. By definition, microscopy has no quantitative value in sub-patent infections and molecular methods will need to be used to characterize the gametocytaemia-infectivity curve at low gametocyte densities.

Despite being the most commonly used amplification target for estimating mature *P. falciparum* gametocytes counts, the use of *Pfs25* transcripts for quantifying gametocyte densities has drawbacks. *Pfs25* mRNA levels are much lower in male compared to female gametocytes [11]. Although it is generally assumed, due to the female bias in sex ratios (4-5:1) in natural infections, that female gametocyte levels are a good surrogate for all-sex gametocytes counts, quantification of the less abundant

male gametocyte-specific *Pfs230p* mRNA or other male targets would provide more accurate estimates of the total number of mature gametocytes as well as sex ratios [11], a factor known to influence infectiousness [37]. In addition to these targets, transcribed only in mature gametocytes, transcripts of other genes have been used for the study of sexual stage malaria: an alternative marker of female gametocytes is *pfg377*, the expression of which begins in the sequestered stage III [38]; a traditional marker for commitment to gametocytogenesis is *Pfs16* [12, 39], which is present in all gametocyte stages. Another early marker of commitment is *PfGEXP5*, the expression of which starts in ring-stage parasites committed to gametocytogenesis, is a potentially useful tool to be used in studies aiming to identify factors influencing commitment to sexual stage [40]. For all of these targets, transcript detection is possible both by the more convenient QT-NASBA and by the more laborious but more reproducible qRT-PCR. Design of intron-spanning primers may close the gap between the attractiveness of both methods and retain the advantage of the more reproducible qRT-PCR [41].

Conclusion

Estimating the contribution of sub-microscopic infections to malaria transmission is a priority in the malaria elimination era. Molecular methods that can quantify gametocytes levels in microscopically undetectable infections come with a promise to improve understanding of malaria epidemiology [42]. qRT-PCR of *Pfs25* mRNA is more reproducible compared to QT-NASBA. Gametocyte densities estimated by both methods, and by microscopy, correlate well with infectiousness in untreated individuals. Although this analysis of field samples suggest that these assays are suitable to quantify patent gametocyte levels, their use to understand the detectability and infectivity of sub-microscopic gametocyte densities is even more important. Future studies thus need to be designed to include individuals with sub-patent gametocyte levels to confirm the sensitivity, precision and relative merits of these assays. Precise quantification of gametocytes at very low levels is of relevance to determine if a threshold density is associated with infectivity provided molecular targets are informative of gametocyte viability and not merely of density [43]. Importantly, neither of the two molecular methods is routinely used in areas where field studies are undertaken and both assays are highly dependent on sample RNA quality.

Declarations

Ethics approval and consent to participate

The study in Mali was approved by the Ethics Committee of the Malaria Research and Training Centre Faculty of Medicine, Pharmacy and Dentistry of the University of Science, Techniques and Technologies of Bamako, and the Committee on Human Research at the University of California, San Francisco (UCSF) and was registered with ClinicalTrials.gov, number NCT01743820 [20]. The study in Burkina Faso was approved by the London School of Hygiene and Tropical Medicine ethics committee, and the *Comité d’Ethique pour la Recherche en Santé (Ministère de la Santé du Burkina Faso)* and was registered with ClinicalTrials.gov, number NCT01935882 [21].

Availability of data and material

The datasets analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors’ contributions

HEP and BPG contributed equally to the completion of this work, taking part in interpretation and analysis of data as well as writing. AD, IC, JoelleB, TB, and RG contributed to the study design for the trial in Mali. AD and SFT oversaw the data collection in Mali. HD, AM, HMS, and IB contributed to the conduct of the study and the data collection in Mali. BPG, IN, ABT, SBS, TB, and CD took part in coordinating the trial in Burkina Faso. KL completed the laboratory work, taking part in the development of methodologies. JohnB contributed to the analysis and interpretation

of data. RB provided the facilities for the laboratory work and took part in useful discussions on data interpretation. IF provided critical assessment and input on the content of this work during completion process. TB contributed to and supervised interpretation and analysis of data, as well as writing. All authors have approved the final version of the manuscript.

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Appendix D, Primaquine pharmacokinetics in African children

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Principal Supervisor	Chris Drakeley
Thesis Title	The human infectious reservoir of falciparum malaria

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Title: Age, weight, and *CYP2D6* genotype are major determinants of primaquine pharmacokinetics in African children

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Abstract

Low dose primaquine is recommended to prevent *Plasmodium falciparum* malaria transmission in areas threatened by artemisinin resistance and areas aiming for malaria elimination. Community treatment campaigns with artemisinin-based combination therapy in combination with the gametocytocidal primaquine dose target all age groups but no studies thus far have assessed the pharmacokinetics of this gametocytocidal drug in African children. We recruited forty children participating in a primaquine efficacy trial in Burkina Faso to study primaquine pharmacokinetics. These children received artemether-lumefantrine and either a 0.25 or a 0.40 mg/kg primaquine dose. Seven blood samples were collected from each participant for primaquine and carboxy-primaquine plasma levels determinations: one sample was collected before primaquine administration and six, after, according to partially overlapping sampling schedules. Physiological population pharmacokinetic modelling was used to assess the impact of weight, age and *CYP2D6* genotype on primaquine and carboxy-primaquine pharmacokinetics. Despite linear weight normalized dosing, the areas under the plasma concentration – time curves and the peak concentrations for both primaquine and carboxy-primaquine increased with age and body weight. Children who were *CYP2D6* poor metabolizers had higher levels of the parent compound, indicating lower PQ *CYP2D6*-mediated metabolism. Our data indicate that primaquine and carboxy-primaquine pharmacokinetics are influenced by age, weight and *CYP2D6* genotype and suggest that dosing strategies may have to be re-considered to maximize the transmission-blocking properties of primaquine.

Introduction

Since 2012, the World Health Organisation (WHO) recommends the use of a single 0.25 mg/kg dose of primaquine (PQ) in combination with standard artemisinin-based combination therapy (ACT) for the treatment of *Plasmodium falciparum* malaria in elimination and resistance containment settings [1]. The rationale for using PQ is to prevent transmission of malaria to mosquitoes as it is the only currently available antimalarial that accelerates the clearance of mature gametocytes post-ACT [2]. Several recent trials assessed the efficacy of the WHO-recommended dose and concluded that it reduces gametocyte carriage compared to ACT alone and effectively prevents transmission in mosquito infection experiments [3-6]. In addition to its use to prevent *P. falciparum* transmission as single dose treatment, PQ has been used for decades in multiple-dose regimens for clearance of *Plasmodium vivax* hypnozoites [7, 8].

The parent compound is not responsible for PQ effects on *P. vivax* hypnozoites [9] and *P. falciparum* gametocytes [10], and the drug metabolising cytochrome P450 2D6 (CYP2D6) enzyme has been implicated in the formation of unknown active metabolites that are responsible for the pharmacological effect of PQ [9, 11-13]. In mice, knocking out the *CYP2D* locus can reduce the metabolism of PQ into its active metabolite against *P. berghei* [11], and increase the area under the plasma concentration – time curve for PQ [14]. The gene coding for this enzyme (*CYP2D6*) is hypervariable in humans and there is limited knowledge on the effects of the variation at this locus on the pharmacokinetics of PQ in humans [9]. Early pharmacokinetic studies of PQ in adults [15-19] identified the main PQ metabolite, carboxy-PQ (C-PQ), which is slowly eliminated and is present at plasma concentrations up to 10 times higher than those of its parent compound [16]. C-PQ is produced by monoamine oxidase (MAO)-A [12], an enzyme involved in drug metabolism in the liver [20], and indirect evidence [9, 21] suggests that it is not the active metabolite against malaria parasites or one of its precursors.

To date, there are only limited PQ pharmacokinetic data [16-18, 22]. This is particularly evident for single low dose PQ and for pharmacokinetic data in children: only one study, undertaken in Papua New Guinea and using single PQ doses of 0.5 or

1.0 mg/kg, recruited children [22]. The difficulty to accurately dose children by extrapolating dosing schemes from adults [23-26] was previously illustrated for the antimalarials sulphadoxine-pyrimethamine and dihydroartemisinin-piperaquine [27]. Since children are frequently infectious to mosquitoes [28] and comprise an important part of the human infectious reservoir for malaria [29], data on single low dose PQ pharmacokinetics in children are highly needed for the planning of community treatment campaigns with PQ to reduce *P. falciparum* transmission.

To identify factors that impact PQ pharmacokinetics in children, we have performed a pharmacokinetic study of PQ in the largest paediatric population thus far.

Materials and Methods

Study site, approvals and patients

A randomised placebo-controlled trial to assess the effect of low dose PQ on malaria transmission was undertaken in Balonghin, a village with endemic malaria transmission in Burkina Faso. Study procedures and results were described in detail elsewhere [4]. Briefly, parasitaemic children aged between 2 and 15 years without malaria symptoms (no measured fever, reported fever or anaemia) and with normal glucose-6-phosphate dehydrogenase (G6PD) enzyme activity were recruited and treated with artemether-lumefantrine (AL) alone, AL and a 0.25 mg/kg PQ dose or AL and a 0.40 mg/kg PQ dose. PQ dosing was achieved by crushing a 15 mg PQ tablet and preparing 1 mg/mL solution by dissolving the crushed tablet in 15 mL of water. This allowed precise dosing: the mean difference between the actual dose given and the assigned dose was -0.004 (95% confidence interval -0.006 – -0.001) mg of PQ/kg of body weight for participants included in this pharmacokinetic study. AL was given twice daily over three days, and PQ or placebo was administered with the fifth AL dose. A subset of study subjects not included in mosquito membrane feeding experiments was invited to participate in the pharmacokinetic study. To minimise the number of blood samples taken per participant whilst maximizing the number of time-points with information on PQ and C-PQ levels, partially overlapping sampling schedules were designed and sampling times were sequentially allocated to participants. The exact time when each blood sample was collected was recorded and used in pharmacokinetic analyses. A total of seven 1.5 – 2 mL venous blood samples

were collected for each study subject: one sample before PQ or placebo administration, four in the first 12 hours following this dose, and two between 24 and 72 hours. All samples were centrifuged within two hours of collection and plasma was subsequently stored at -80°C. Forty participants, 20 from each PQ study arm, had PQ and C-PQ plasma levels quantified and were included in this analysis.

The study was registered at ClinicalTrials.gov (reference number NCT01935882). Written informed consent was obtained for participation in the pharmacokinetics sampling. The clinical trial received ethics approval from the London School of Hygiene and Tropical Medicine ethics committee (reference number 6274), and the Comité d'Ethique pour la Recherche en Santé (Ministère de la Santé du Burkina Faso; reference number 2012-10-78).

Quantification of PQ and C-PQ plasma levels

PQ and C-PQ levels were determined by liquid chromatography/mass spectrometry (LC-MS) as previously described [30]. The system consisted of a Shimadzu LCMS-2010A mass spectrometer operated using electrospray ionization (ESI) in positive ion detection mode. Data were collected in the selected ion monitoring mode at 325.35 m/z for quinine (internal standard, retention time 3.7 minutes), 260.30 m/z for PQ (retention time 5 minutes) and 275.25 m/z for C-PQ (retention time 8 minutes). The analytical column was a Phenomenex Synergi Polar RP (150 x 2 mm, 4u), preceded by a Phenomenex Polar RP security guard column (2 x 4mm, Torrance, CA). The standard curve ranged from 4 to 1,000 ng/mL, with a lower limit of quantitation of 4 ng/mL and a lower level of detection of 1 ng/mL. All control values were within 15% of their nominal value.

CYP2D6 genotyping

EDTA-anticoagulated venous blood and/or saliva samples collected with Oragene kit (OG-500 or OG-575) were used as sources of human genomic DNA for *CYP2D6* genotyping. DNA was extracted using a MagNAPure LC automated extractor and extraction kits for large volume samples according to manufacturer's instructions. DNA concentration was measured fluorometrically using a Qubit fluorometer and accompanying high sensitivity (HS) kit. Samples were diluted according to manufacturer's instructions for assays determining copy number variation (CNV) and

for preamplification as well as sequence variant determination with QuantStudio 12K Flex OpenArrays with TaqMan assays. OpenArray analysis was repeated for five samples without preamplification, due to undetermined genotype. In total, two CNV assays (hs00010001_cn targeting exon 9 and hs04083572_cn targeting intron 2 of *CYP2D6*) and 19 sequence variants in *CYP2D6* were analysed (see Supplemental Material **Table S1** for assay details) and genotype was determined according to the cytochrome P450 allele nomenclature website [31]. Inferred phenotype was determined using the Activity Score (AS) [32].

Pharmacokinetic modelling

A physiological population pharmacokinetic model was developed to allow better extrapolation and enable identification of pharmacokinetic parameters that cannot be identified in classical empirical models [33]. Pharmacokinetic analysis was performed by means of non-linear mixed effects modelling with the software NONMEM V.7.3.0, and Piraña as an interface for NONMEM, R-statistics and Perl Speaks Nonmem V4.6.0 [34]. The covariance step in NONMEM was used to calculate parameter precision. To account *a priori* for changes in pharmacokinetics related to growth, liver volume (V_L) was calculated from total body weight and height [35]. All other volumes and flow parameters were allometrically scaled to a total body weight of 70 kg, as previously proposed. The exponents of the allometric models were fixed at 0.75 and 1 for flow and volume parameters, respectively [36]. Due to the high co-linearity of age and weight, this enabled to separately assess the impact of age and weight on PQ pharmacokinetics. The PQ dose and measured plasma concentrations of PQ and C-PQ were converted to their molar equivalents for this analysis. Parameter shrinkage, with a shrinkage of >25% indicating uninformative data to estimate the parameter [37], was derived from the NONMEM results file.

As PQ is thought to be mainly metabolised by MAO and cytochrome P450s in the liver [12], a well-stirred liver model, a well-established model to describe hepatic metabolism of drugs, was implemented to describe the physiologically plausible relationship between first-pass and central metabolism [38, 39]. Apparent intrinsic hepatic clearances for MAO- and CYP2D6-mediated metabolism ($CL_{int,MAO}$ and $CL_{int,CYP2D6}$, respectively) were estimated. C-PQ was assumed to originate from the MAO-mediated metabolism of PQ. The individual CYP2D6 intrinsic clearance

($CL_{int,CYP2D6,i}$) was calculated from the population intrinsic clearance ($CL_{int,CYP2D6,pop}$) and CYP2D6 AS with the formula

$$CL_{int,CYP2D6,i} = AS \times CL_{int,CYP2D6,pop}$$

We assumed a liver plasma flow (Q_H) of 49.5 L/h, derived from an adult total blood flow of 90 L/h and a plasma fraction of 55% in whole blood (haematocrit level of 45%). The hepatic extraction (E_H) was defined as

$$E_H = CL_{int}/(Q_{HP} + CL_{int})$$

and the apparent MAO- and CYP2D6-mediated hepatic clearances ($CL_{H,MAO}$ and $CL_{H,CYP2D6}$) were calculated using the formula

$$CL_H = E_H \times Q_{HP}$$

Gradual onset of oral drug absorption was described with a chain of transition compartments, as described earlier [40]. In short, the mean absorption time (MAT) was estimated and the rate constant (k_{tr}) for these transition compartments was calculated using

$$k_{tr} = (n + 1)/MAT$$

where n equals the number of transition compartments. The inter-individual variability was modelled by means of an exponential variance model. Throughout model building, basic goodness-of-fit plots and prediction-corrected visual predictive checks [41] were explored. Concentrations that were below the limit of quantification were retained in the analysis employing the M6 method, as proposed by Beal [42] and, therefore, the first concentrations below the limit of quantification (BLOQ) were fixed to $\frac{1}{2}$ LOQ and a fixed residual additive error of $\frac{1}{2}$ LOQ was introduced in the model. Individuals with missing or inconclusive *CYP2D6* genotype data (N=4) were retained in the model by imputing the individual activity scores using mixture modelling, based on the frequencies observed in the study population, as proposed earlier [43]. The Bayesian imputed activity score was estimated to be 1.5 for all four children. As fixing the individual activity scores manually resulted in better model stability and no significant change of model goodness-of-fit, in the final model these missing activity scores were manually set to 1.5. The final model was used to obtain the empirical Bayes estimates for the area under the concentration – time curve (AUC) to infinity, the maximum concentration (C_{max}), and the time of C_{max} (T_{max}) for both PQ and C-PQ.

Results

Study population

40 afebrile children aged 2 to 14 years who received a single low dose (0.25 or 0.40 mg/kg) of PQ on the final day of the 6-dose AL regimen were included in this study. 37/40 had patent asexual stage *P. falciparum* parasites at enrolment (median and interquartile range [IQR] 1,252 [578-2,503] parasites per μL). Median (IQR) haemoglobin level at enrolment was 11.6 (10.8 – 12.5) g/dL and similar in the two study arms. **Table 1** summarises demographics and baseline laboratory results for these 40 children.

Table 1. Baseline Characteristics

	Study arm	
	<i>0.25 mg/kg PQ</i>	<i>0.40 mg/kg PQ</i>
<i>Number of participants</i>	20	20
<i>Gender (% female)</i>	55	60
	Median (IQR [‡])	Median (IQR)
<i>Age (years)</i>	8 (6 - 10)	10 (6.5 - 12)
<i>Body weight (kg)</i>	19.8 (16.2 - 26.3)	24.9 (15.9 - 31.6)
<i>Height (cm)</i>	118 (104 - 133.5)	129 (104 - 144.5)
<i>Temperature (°C)</i>	36.6 (36.4 – 37.0)	36.7 (36.1 – 36.9)
<i>Asexual parasites (per µL)</i>	991 (635.5 - 2066.5)	1188 (284.5 - 3340)
<i>Haemoglobin (g/dL)[□]</i>	11.4 (10.6 - 12.5)	11.9 (11.1 - 12.7)
<i>Alanine transaminase (U/L)</i>	22.5 (17.5 - 34.5)	22 (17 - 29)
<i>Aspartate transaminase (U/L)</i>	38 (32 - 46)	36 (27 - 41)
<i>Total bilirubin (µmol/L)</i>	7.4 (5.4 - 9.9)	10.8 (8 - 13.8)
<i>Creatinine (µmol/L)</i>	37.8 (35.5 - 40.8)	38.4 (32.7 - 45.2)

[‡]*Interquartile range*

[□]*Haemoglobin levels measure by Hemocue*

CYP2D6 genotyping

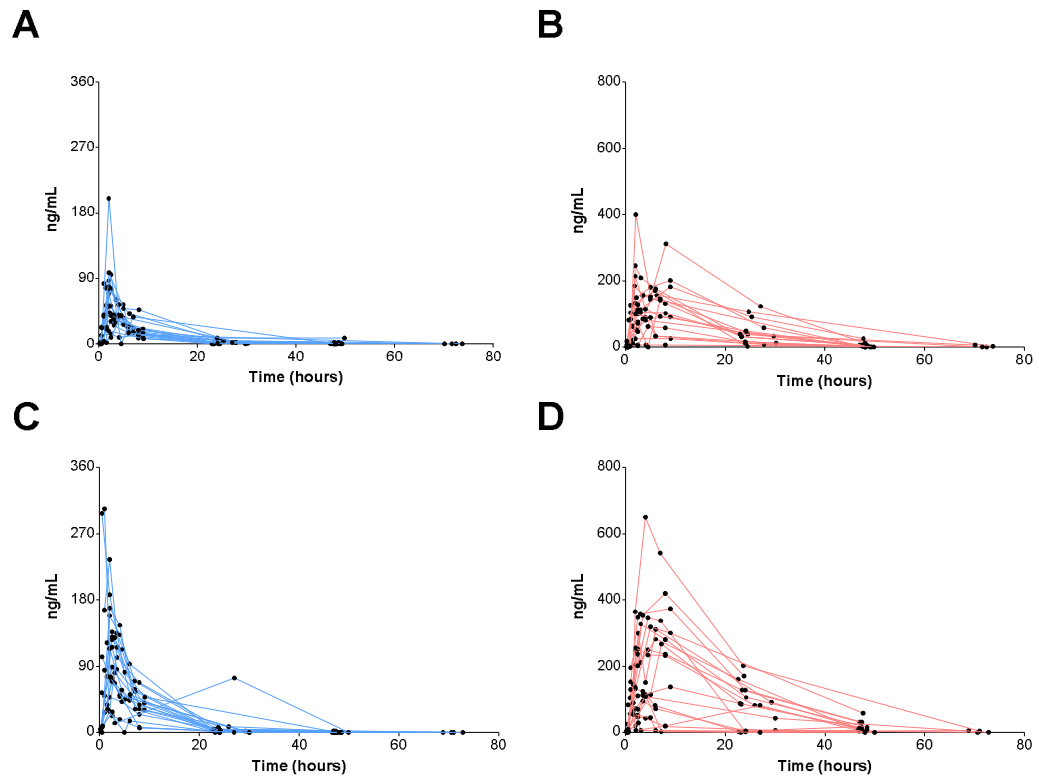
CYP2D6 genotyping was successful for 36/40 children. For 3/40 participants, no samples were available for genotyping, and for one, genotyping was inconclusive. Allele frequencies are presented in the Supplemental Material (**Table S2**). 1/36 (2.8 %), 10/36 (27.8 %), 22/36 (61.1 %) and 3/36 (8.3 %) study subjects were classified as poor metabolizer (PM, AS of 0), intermediate metabolizer (IM, AS of 0.5 or 1.0), extensive or normal metabolizer (EM, AS of 1.5 or 2.0) and ultrarapid metabolizer (UM, AS of 3.0), respectively [44, 45].

Pharmacokinetics

A total of 274 plasma samples were collected. Two of the 40 children were excluded from the pharmacokinetic analysis because it was not possible to determine PQ and C-PQ levels in their samples due to inadequate sample volume.

The raw pharmacokinetic data of PQ and C-PQ per dose group are depicted in **Figure 1**. As observed, PQ was rapidly absorbed and plasma C-PQ concentrations were generally higher than plasma PQ concentrations. Overall, the pharmacokinetics of the parent compound and of its main plasma metabolite presented substantial inter-individual variation.

Figure 1. PQ and C-PQ plasma levels (y-axes) after PQ administration (x-axes). In A and C, PQ levels are presented for participants who received the 0.25 and 0.40 mg/kg PQ dose, respectively. In B and D, C-PQ levels are presented for the 0.25 and 0.40 mg/kg PQ study arms, respectively. Assay results of all samples collected after PQ administration, including those with PQ or C-PQ levels below the limit of detection (i.e. with assigned level of 0 ng/mL), are presented.



A physiological pharmacokinetic model was developed to estimate pharmacokinetic parameters for both PQ and C-PQ (**Figure 2**). First order kinetics with two absorption transit compartments and one compartment disposition for PQ and C-PQ fit the observed plasma levels well. PQ was rapidly absorbed with a mean absorption time of 0.706 h (relative standard error [coefficient of variation] 12%). Although allometric clearance appeared to explain most weight-related variability in pharmacokinetics, over-prediction of plasma PQ and C-PQ concentrations was observed in the youngest children of our study population. This resulted in higher than expected estimates for apparent volume of distribution and apparent clearance in these children. This phenomenon may be explained by a reduced relative bioavailability at younger age. Therefore, maturation of relative bioavailability (F) with age (i.e., the increase in bioavailability with age) was described by an E_{max} model with the formula

$$F = \text{Age} / (\text{Age} + F_{50})$$

where F_{50} is the age in years at which the relative bioavailability is 50% that of the mature value. F_{50} was estimated to be 4.27 years (relative standard error 44%), explained all observed inter-individual variability in relative bioavailability, significantly ($P < 0.001$) improved model fit and was, therefore, retained in the model. In addition to apparent age-dependent bioavailability, the inclusion of CYP2D6-mediated clearance of PQ, assumed to be linearly related to the CYP2D6 activity score, significantly improved model fit ($p < 0.001$) and was also retained in the final model. Diagnostic prediction-corrected visual checks of the model are shown in **Figure 3** and additional goodness-of-fit assessments are presented in the Supplemental Material (**Figures S1 and S2**). Estimated model parameters and their variability are presented in **Table 2**.

Figure 2. Schematic representation of the model. The mass transport of this model can be described with the following rate constants:

$$k_{12} = 3/\text{MAT}$$

$$k_{23} = 3/\text{MAT}$$

$$k_{34} = 3/\text{MAT}$$

$$k_{40} = CL_{H, \text{CYP2D6}}/V_L$$

$$k_{45} = CL_{H, \text{MAO}}/V_L$$

$$k_{50} = CL_{\text{CPQ}}/V_{\text{CPQ}}$$

$$k_{46} = (Q_H (1-E_H))/V_L$$

$$k_{64} = Q_H/V_{\text{PQ}}.$$

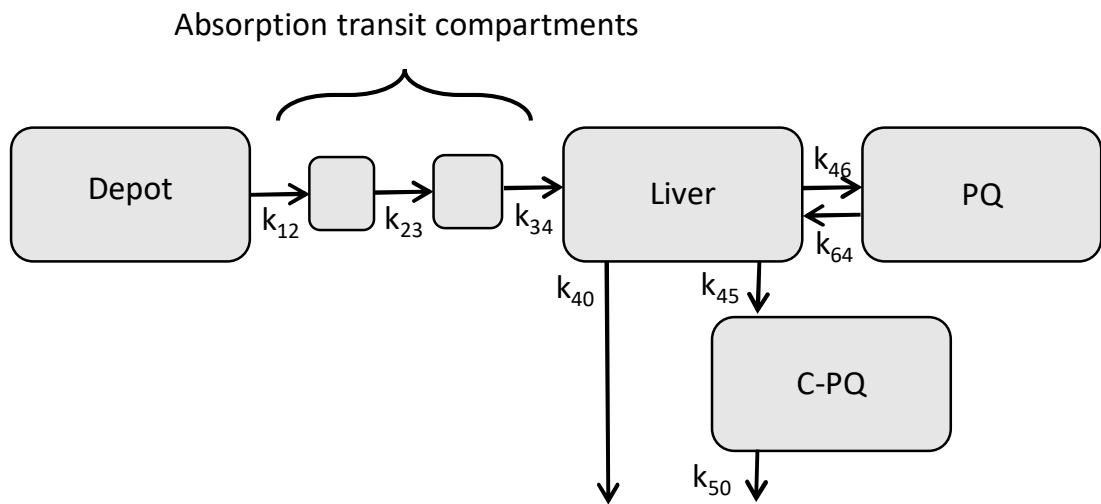


Figure 3. Prediction-corrected visual predictive check of observed data. Left and right panels depict the prediction-corrected visual predictive checks for PQ and C-PQ, respectively, based on 1000 simulations. Prediction-corrected simulated (shaded areas) and observed (circles and lines) PQ and C-PQ concentrations are presented over time (h; y-axes). The thick red line connects the observed median values per bin. The dotted red lines connect the 5th and 95th percentiles of the observations. The light blue areas are the 95 % confidence interval of the 5th and 95th percentiles, and the light red area indicates the confidence interval of the median.

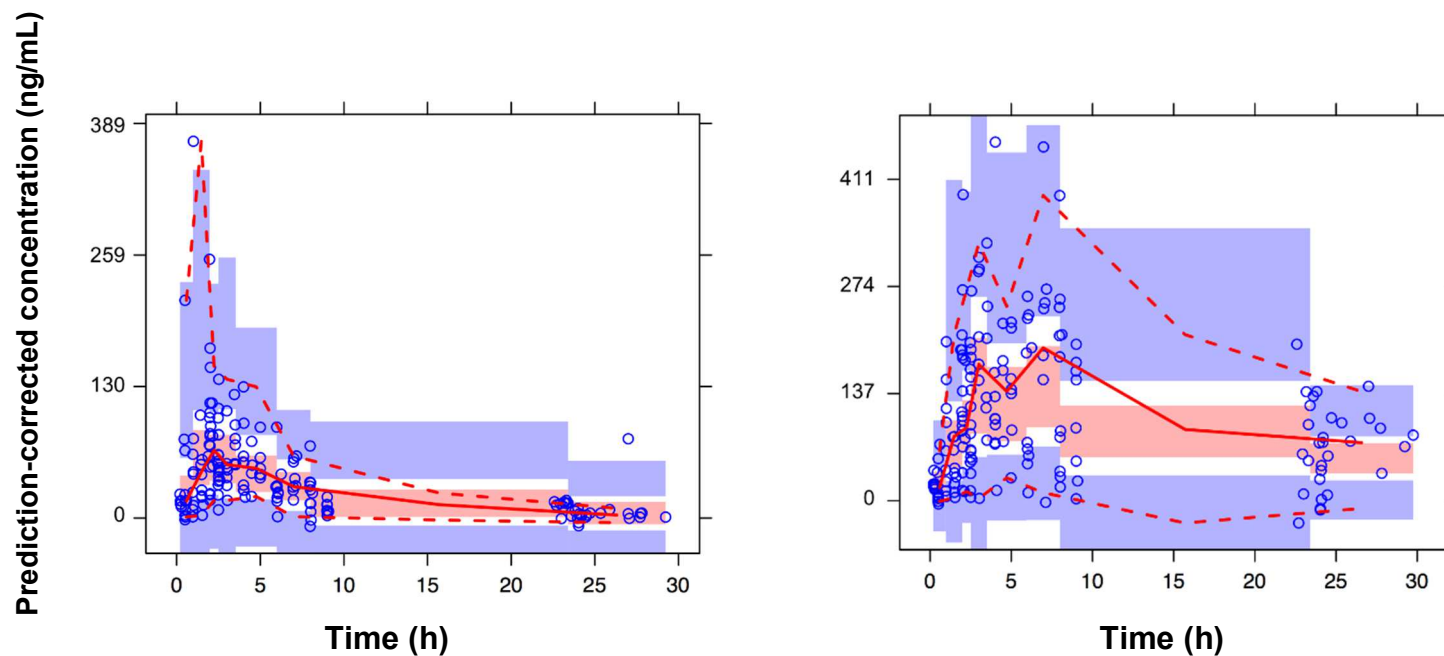


Table 2. Pharmacokinetic model parameters. All flow and volume parameters were allometrically scaled to a body weight of 70 kg with an allometric exponent of 0.75 for flow parameters and an exponent of 1 for volume parameters. CV = coefficient of variation.

Parameter	Estimate	Relative standard error of estimate (% CV)	Shrinkage (%)
Mean absorption time (<i>MAT</i>)	0.706 h	12%	-
PQ volume of distribution (<i>V_{PQ}</i>) (70kg)	127 L	20%	-
Inter-individual variability <i>V_{PQ}</i> (%)	82.9%	38%	12.2%
C-PQ volume of distribution (<i>V_{CPQ}</i>) (70 kg)	21.7 L	39%	-
<i>CL_{int,MAO}</i> (70 kg)	7.35 L/h	41%	-
Inter-individual variability <i>CL_{int,MAO}</i> (%)	65.3%	33%	13.1%
<i>CL_{int,CYP2D6,pop}</i> (70 kg)	6.70 L/h	69%	-
<i>CL_{CPQ}</i> (70 kg)	1.50 L/h	35%	-
<i>F₅₀</i>	4.27 y	44%	-
Residual error PQ Proportional Additive	32.7% 2 ng/mL (FIX)	42%	19.5%
Residual error CPQ Proportional Additive	45.2% 2 ng/ml (FIX)	20%	21.6%

FIX: values were fixed during modelling

Model-derived median (range) AUC , C_{max} and T_{max} for PQ were 600.26 (259.87 – 3,315.40) h x ng/mL, 68.42 (20.21 – 391.16) ng/mL and 1.59 (0.96 – 2.12) h, respectively, and are in agreement with previous studies [18]; for C-PQ these values were 3,468.35 (962.05 – 10,506) h x ng/mL, 147.23 (25.61 – 403.95) ng/mL and 6.80 (2.73 – 16.03) h, respectively. The 0.40 mg/kg PQ dose was associated with higher AUC and C_{max} compared to the 0.25 mg/kg dose (**Table 3**), although there was substantial variation within each study arm: C_{max} estimates included values that were 6 – 10 times higher than the lowest model-derived C_{max} in each study arm; a similar pattern was observed for AUC .

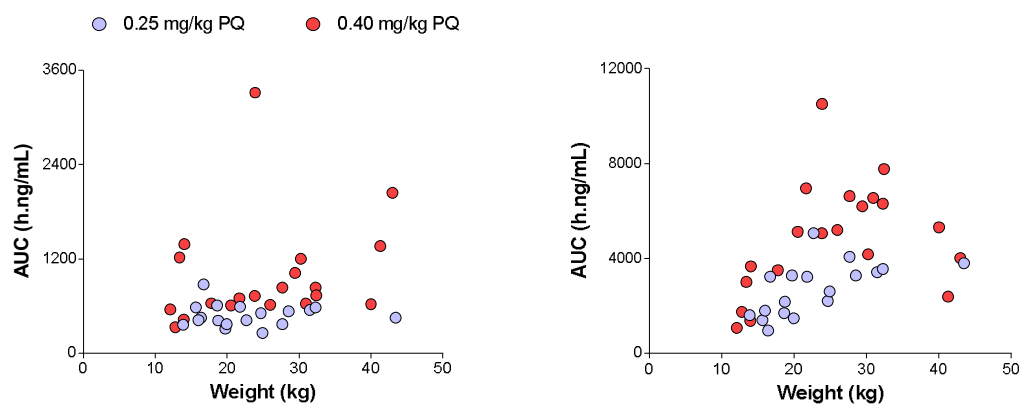
Table 3. Model-derived pharmacokinetic parameters for PQ and C-PQ in African children receiving a single PQ dose of 0.25 (n=18) or 0.40 mg/kg (n=20). Values are reported as medians (minimum – maximum).

Parameters	0.25 mg/kg PQ		0.40 mg/kg PQ	
	PQ	C-PQ	PQ	C-PQ
T_{max} (h)	1.6 (1.2 – 1.9)	7.1 (4.3 – 10.1)	1.6 (1.0 – 2.1)	6.6 (2.7 – 16.0)
C_{max} (ng/ml)	50.2 (20.2 – 138.7)	108.3 (25.6 – 240.2)	88.9 (24.4 – 391.2)	196.8 (29.3 – 404.0)
$AUC_{0-\infty}$ (h x ng/mL)	450.4 (259.9 – 875.9)	2912.3 (962.1 – 5076.3)	730.7 (334.2 – 3315.4)	5091.7 (1075.0 – 10506)

Host characteristics influencing PQ pharmacokinetics

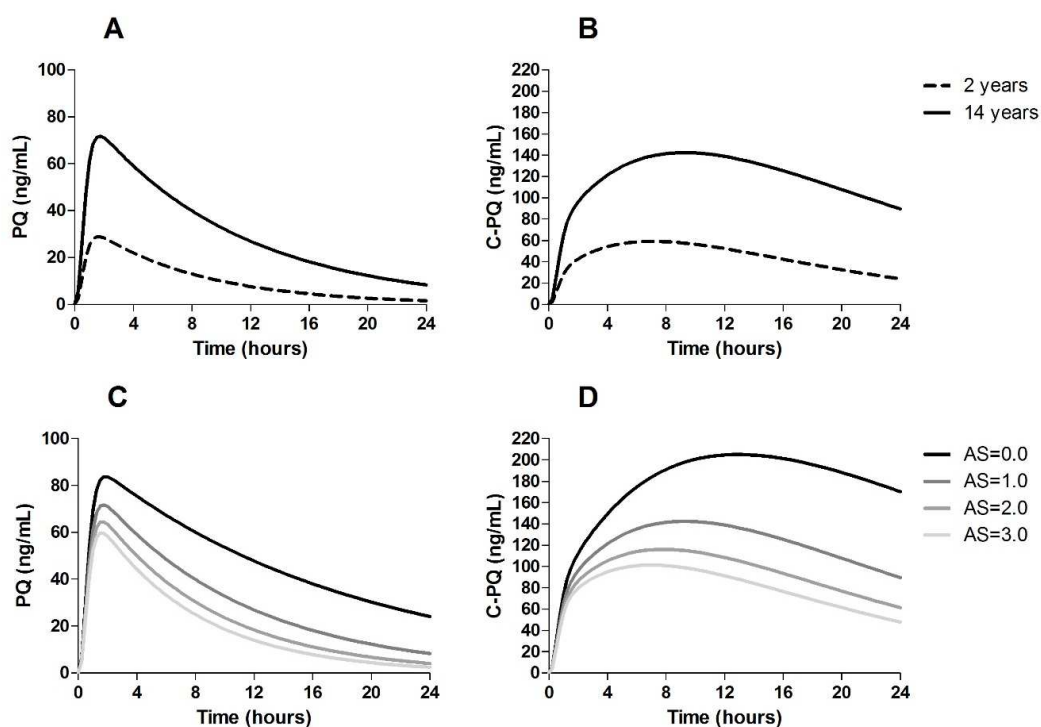
PQ doses are linearly scaled with body weight. To assess whether this approach is appropriate, we analysed plasma PQ and C-PQ concentrations by weight. In **Figure 4**, the distribution of model-derived *AUC* values by weight and PQ dose is presented. For each dose, despite linear dose extrapolation based on weight, *AUC* estimates of PQ and C-PQ were positively correlated with body weight. A similar pattern was observed when analysing the relationship between *AUC* values and age (Supplemental Material, **Figure S3**).

Figure 4. Area under the plasma concentration – time curve for both PQ (left panel) and C-PQ (right panel) by weight (x-axes).



CYP2D6 AS was an important determinant of PQ and C-PQ concentrations. In **Figure 5**, typical plasma PQ and C-PQ concentration – time curves for children aged 2 (body weight 12 kg) and 14 (body weight 40 kg) years old and with different CYP2D6 metabolizer status are presented. Toddlers who are IM have particularly low plasma levels of PQ compared to schoolchildren with PM status.

Figure 5. Effect of age and CYP2D6 Activity Score (AS) on PQ and C-PQ plasma concentrations over time after 0.25 mg/kg single dose of PQ. Panels A and B respectively show the effect of age on PQ and C-PQ concentrations over time, in two hypothetical children of different ages (2-year-old 12kg and 14-year-old 40 kg body weight), with CYP2D6 AS=1.0. Panels C and D respectively show the effect of genetically determined CYP2D6 AS, in four hypothetical 14-year-old children, on PQ and C-PQ concentration over time. The values use to construct curves are model derived.



Discussion

The use of PQ to prevent *P. falciparum* transmission may support malaria elimination activities and efforts to contain artemisinin resistance. Understanding PQ pharmacokinetics is important to optimise dosing regimen aimed at clearing gametocytes and reducing infectivity to mosquitoes. We performed the first PQ pharmacokinetic study in African children and observed that age, body weight and *CYP2D6* genotype influenced PQ and C-PQ plasma levels: younger children and children with lower body weight have lower levels of PQ and C-PQ, while poor *CYP2D6* metabolizers have higher levels of PQ. These findings indicate that linear weight-based dosing may be sub-optimal to achieve efficacious PQ concentrations.

Since the 1980s several PQ pharmacokinetic studies have been performed; however only one of these studies enrolled children [22]. This is the first PQ pharmacokinetic study recruiting African children, a group that may represent 28.4 – 51.8% of all individuals capable of infecting mosquitoes in some endemic areas [28] and thus are an important target population for PQ treatment. Our analysis generated several insights into PQ pharmacokinetics in this age group. First, both age and weight explain variability in PQ pharmacokinetics in children and show a non-linear relationship with PQ exposure. This is illustrated by the clear relationship between age and PQ exposure despite linear dose normalization based on weight. Therefore, linear dosing based on total body weight is inappropriate to obtain similar drug exposure in children as in adults, confirming earlier findings that (non-linear) allometric scaling of pharmacokinetics of PQ accounted for the observed variability in Papua New Guinean children [22]. However, this study did not report an age effect on bioavailability as observed by us. This might be explained by the fact that in our study PQ was administered using 1 mg/mL solutions, which allows for more accurate dosing than tablet-based regimens, and that our study population covered a wider age range (2 – 14 years) compared to the previous study in Papua New Guinea (6 – 10 years). The physiological cause of the observed maturation of bioavailability remains unclear. This phenomenon is not uncommon in paediatric pharmacokinetics: for example, the bioavailability of the liquid formulation of the antiretroviral drug efavirenz also matured with age in young children [46]. Potential causes could be age-related changes in gastrointestinal motility, pH or pre-hepatic expression of metabolic

enzymes or transporters, possibly resulting in increased absorptive capacity with age [47, 48]. These findings should be confirmed prospectively with additional PQ pharmacokinetic and pharmacodynamic studies in paediatric as well as adult populations to assess the effect of weight and age on PQ-related pharmacokinetics as well as gametocyte clearance, transmission reduction and toxicity.

Another important finding of our study is that *CYP2D6* genotype influences PQ pharmacokinetics [14]. Initial PQ concentrations are affected by *CYP2D6* genotype since CYP2D6-mediated metabolism occurs both systemically and pre-systemically. The influence of CYP2D6 metabolizer status on PQ efficacy was previously shown by an increased *P. vivax* relapse rate in individuals with *CYP2D6* PM and IM genotypes [9]. Individuals with higher relapse rates, presumably because of an inability to clear *P. vivax* hypnozoites, had a significantly higher PQ *AUC* than extensive metabolizers but no differences in C-PQ pharmacokinetics parameters, which suggests that CYP2D6 activity is a rate-limiting step in the formation of active metabolite(s) against *P. vivax* hypnozoites [9, 11]. It has been hypothesized that unknown metabolites formed through CYP2D6 are also responsible for the effect of PQ on *P. falciparum* transmission, however direct evidence for this is lacking and available field studies with comprehensive CYP2D6 data are too small to assess the effect of CYP2D6 metabolizer status on gametocytaemia and transmission potential after single low dose PQ. CYP2D6-related differences in PQ transmission-blocking efficacy would be particularly relevant in malaria elimination settings, where mass drug administration are used to accelerate transmission interruption: CYP2D6 poor metabolizers might remain infectious for longer periods of time after PQ administration compared to individuals with other *CYP2D6* genotypes and could represent a source of residual malaria transmission in these areas, depending on the frequency of alleles linked to this phenotype in the population.

Since PQ metabolism is considered essential to its effect on falciparum transmission [10], one may argue that exposure to the parent drug (PQ) is not important. However, as formation of active metabolites depends on the presence of parent drug, one should aim for adequate initial exposure of PQ. Our findings suggest that age, body weight and *CYP2D6* genotype can all influence PQ levels and consequently may determine the levels of active metabolites generated. These observations and the fact that

currently available PQ tablet sizes are not optimal for paediatric dosing, posing further challenges in achieving the target PQ dose in children, indicate that PQ dosing strategies may have to be re-considered. The therapeutic range over which PQ prevents malaria transmission is currently not well established but may include doses lower than the WHO-recommended PQ dose of 0.25 mg/kg [5]. PQ efficacy and added value over ACT alone may also depend on the ACT used [4, 5, 49]. The gametocytocidal and transmission blocking effects of AL are superior to that of dihydroartemisinin-piperaquine but there are concerns for drug-drug interactions between AL and PQ. Indeed, although artemether does not influence PQ metabolism [50], lumefantrine inhibits CYP2D6 *in vitro* [51, 52]. In our study, although participants were treated with lumefantrine, we found that CYP2D6 Activity Score explained variability in PQ pharmacokinetics, indicating that if CYP2D6 was indeed inhibited by lumefantrine, this inhibition was incomplete at the time of PQ administration. Since AL alone is by far the most widely used ACT, our CYP2D6-related findings are of immediate relevance for ACT-PQ policies and our findings of age-dependent exposure to PQ and C-PQ are likely to be independent of the type of ACT-PQ combination.

PQ is considered a valuable tool to support malaria elimination efforts. Whether it is deployed during mass drug administration campaigns, when all individuals in a community receive treatment, or during treatment of symptomatic falciparum malaria episodes, a substantial proportion of PQ doses are likely to be given to young children. Age-related changes in PQ pharmacokinetics, like maturation of bioavailability or non-linear change in clearance with weight, indicate that dose extrapolation from adult regimens based solely and linearly on weight may not be an optimal approach. A limitation of our study is that the studied population was relatively small and did not include subjects outside the 2 – 14 years range. Extrapolation of our findings outside this range should, therefore, be performed with caution. Future studies relating plasma concentrations of PQ and its (active) metabolites are needed to quantify the implications of our findings for the ability to prevent *P. falciparum* transmission by PQ treatment and to inform better dosing strategies.

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Appendix E, Malaria reservoir in low endemic areas

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SECTION A – Student Details

Student	Bronner Pamplona Augusto Gonçalves
Principal Supervisor	Chris Drakeley
Thesis Title	The human infectious reservoir of falciparum malaria

If the Research Paper has previously been published please complete Section B, if not please move to Section C

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Section B: Paper already published			
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Student Signature: Bronner P. A. G. Date: 04/09/2017

Supervisor Signature: Chris Drakeley Date: 04/09/2017

Title: Infectivity of microscopic and submicroscopic malaria infections in low endemic settings

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The contribution of low density, subpatent *Plasmodium falciparum* to mosquito infection and onward transmission of the disease has received much recent attention as a determinant of the success of malaria elimination strategies. The study by Lin and colleagues [1] addresses this issue in the context of low malaria transmission intensity in Cambodia. The authors observed that 5.9% of adults with symptomatic malaria were infectious in mosquito feeding experiments and that the vast majority of these infections (96%) arose from patients whose blood had microscopically detectable gametocytes. The study provides valuable information on infectivity in this particular group, i.e. adults with clinical malaria attending hospital. It also suggests that areas where individuals seek medical care early in their infection, with fewer mature gametocytes, would have lower infectivity than areas where clinic attendance is delayed, perhaps because symptoms take longer to develop and/or individuals postpone seeking treatment, and gametocyte levels have more time to increase.

Whilst these observations made in the Cambodian study are valuable, there are several important considerations when using these clinic-based observations to draw broader conclusions on the human infectious reservoir for malaria. In many settings, the majority of malaria infections do not elicit effective care seeking behaviour but persist as asymptomatic infections. A recent large epidemiological study in Cambodia, Vietnam and Thailand-Myanmar border showed that 20% of the population in these settings harboured infections, most of which were afebrile [2]. Imwong and colleagues [3] recently demonstrated that the mean density of these asymptomatic infections is approximately 5 parasites per microliter; the majority of infections are thereby present at densities that are unlikely to be detected by microscopy or rapid diagnostic tests [4, 5]. It is the contribution of these asymptomatic and often submicroscopic infections to the infectious reservoir that is poorly characterised.

The conclusion of Lin and colleagues that ultrasensitive diagnostics are not required to identify those who are most infectious to mosquitoes is most probably correct. It is however, still very much an open question what proportion of all mosquito infections is caused by individuals with clinical malaria seeking treatment and what proportion by asymptomatic, chronic infections which may be submicroscopic. Indeed, in an

earlier study in Thailand, Pethleart and colleagues [6] concluded that the infectious reservoir was largely driven by the latter group, that is, infected individuals who did not attend clinics. That study also concluded that submicroscopic gametocytes were an important source of mosquito infections in this population. Moreover, the true relevance of the results from any of the artificial mosquito infections needs to be balanced by natural mosquito dynamics such as local abundance, biting rates and the species-specific susceptibility to infection [7].

Ultimately, where the health system is able to (or can be improved to be able to) rapidly identify and treat infections it seems reasonable to assume that the duration of symptomatic infections will be shortened resulting in fewer infectious individuals. This could have an important impact on overall malaria transmission as suggested in the optimistic conclusions of Lin *et al.* However in many settings in Africa and Asia, asymptomatically infected individuals vastly outnumber those with symptoms and the lack of interaction with the health systems means that many infections remain untreated. It is imperative that health facility identification and treatment of malaria remain the cornerstone of malaria control programmes but specific targeting of asymptomatic infections is likely to accelerate the progress toward elimination. Further studies on the importance of asymptomatic and submicroscopic malaria infections for onward transmission will facilitate this progress.

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Appendix F, Heterogeneity in exposure to *Anopheles* mosquitoes

This is a revised version of the manuscript included in chapter 6. This work was submitted for publication in October 2017 and is currently under review in *eLife*.



RESEARCH PAPER COVER SHEET

PLEASE NOTE THAT A COVER SHEET MUST BE COMPLETED FOR EACH RESEARCH PAPER INCLUDED IN A THESIS

SECTION A – Student Details

Student	Bronner Pamplona Augusto Gonçalves
Principal Supervisor	Chris Drakeley
Thesis Title	The human infectious reservoir of falciparum malaria

If the Research Paper has previously been published please complete Section B, if not please move to Section C

SECTION B – Paper already published

Where was the work published?	
When was the work published?	
If the work was published prior to registration for your research degree, give a brief rationale for its inclusion	
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SECTION C – Prepared for publication, but not yet published

Where is the work intended to be published?	eLife
Please list the paper's authors in the intended authorship order:	Wamdaogo M. Guelbéogo*, Bronner P. Gonçalves*, Lynn Grignard, John Bradley, Samuel Serme, Joel Hellewell, Kjerstin Lanke, Soumanaba Zongo, Nuno Sepúlveda, Issiaka Soulama, Wangrawa Dimitri, Laith Yakob, Sagnon N'Falé, Teun Bousema#, Chris Drakeley#
Stage of publication	Under review

SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper	I wrote the first draft of this manuscript, with TB and CD. I performed the most analyses included in this version. JB contributed to the statistical analysis, and Laith Yakob, to mathematical modelling.
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Student Signature: Bronner P. Gonçalves

Date: 12/12/2017

Supervisor Signature: Chris Drakeley

Date: 12/12/2017

Title: Variation in natural exposure to anopheles mosquitoes and its effects on malaria transmission

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Abstract

Variation in biting frequency by *Anopheles* mosquitoes can explain some of the heterogeneity in malaria transmission in endemic areas. In this study in Burkina Faso, we assessed natural exposure to mosquitoes by matching the genotype of blood meals from 1,066 mosquitoes with blood from residents of local households. We observed that the distribution of mosquito bites exceeded the Pareto rule (20/80) in two of the three surveys performed (20/85, 76, and 96) and, at its most pronounced, is estimated to have profound epidemiological consequences, inflating the basic reproduction number of malaria by 8-fold. The distribution of bites from sporozoite-positive mosquitoes followed a similar pattern, with a small number of individuals within households receiving multiple potentially infectious bites over the period of a few days. Together, our findings indicate that heterogeneity in mosquito exposure contributes considerably to heterogeneity in infection risk and suggest significant variation in malaria transmission potential.

Introduction

Malaria epidemiology is doubly dependent on the frequency and efficiency of contacts between human hosts and *Anopheles* mosquitoes, which link the number of mosquito infections caused by an infectious human host and the rate at which uninfected humans acquire infections. Describing the variability in the frequency of human sampling by malaria vectors is therefore essential to understand parasite transmission from and to humans. While at a local level vector density determines average mosquito exposure, even within the same locality individuals may not be equally likely to be bitten by *Anopheles* mosquitoes (1-4). Exposure to malaria vectors is influenced by host availability (i.e., amount of time an individual remains unprotected against mosquito bites in an environment where anopheline mosquitoes are present) and attractiveness to mosquitoes (5, 6). Availability determines when and where individuals might be sampled by mosquitoes: a multicentre study in Africa that collected entomological and human behavioural data estimated that more than three quarters of human exposure to anopheline mosquito bites occur when individuals are indoors (7). For individuals who are accessible to malaria vectors, age and body surface area (3, 8) are two major determinants of attractiveness to mosquitoes, although other factors also play a role (2).

The multifactorial nature of mosquito exposure in malaria endemic areas indicates that, while experimental and quasi-experimental, e.g. involving modified tents and huts, entomological studies are valuable, they will not accurately capture inter-individual variation in actual exposure. Identifying transmission heterogeneities, especially extreme heterogeneities, is however critical to better inform infectious disease epidemiology and well-established theory has elucidated their implications for pathogen spread and control measures (9, 10). Here, we describe the variability in natural exposure to malaria vectors by linking, through DNA fingerprinting, blood meals of wild-caught mosquitoes to humans living in the households where they were collected. Previously we have shown that these mosquitoes fed more often on adults (11). We now extend this analysis to assess the degree of heterogeneity in the distribution of mosquito bites in the population at different times during the transmission season. We also present the frequency of *Anopheles* species-specific mosquito bites, and potential parasite inoculations (i.e., sporozoite-positive mosquito bites) per individual.

Results

Study households

We performed indoor resting collections of anopheline mosquitoes in an area with seasonal malaria transmission in Burkina Faso. Thirty-five households were included in this analysis. The median number of individuals living in each study household was 3 (range, 2 – 5). Reported bed net use among the study participants was high (111/126, 88.1%). At enrolment (October – December 2013), most (79.2%) individuals were parasite-positive by *18S* qPCR; in 21/35 houses, all individuals were infected. We collected 325, 620 and 190 bloodfed *Anopheles* mosquitoes at the start (2014), peak (2014) and end (2013) of the transmission season, respectively (**Table 1**). During the 2013 survey, 21/35 houses had at least one bloodfed mosquito collected; in 2014, 19/20 and 20/20 households had fed mosquitoes collected at the start and peak of the transmission season, respectively. The average number of bloodfed mosquitoes collected per day in each household was higher at the start (median 2.3, interquartile range [IQR] 0.7 – 5.5) and peak (median 3.9, IQR 2.1 – 8.5) compared to the end (median 0.1, IQR 0 – 0.6 mosquitoes per day) of the transmission season ($P = 0.001$ and < 0.001 , respectively). There was no correlation between the average number of bloodfed mosquitoes collected per day and the number of individuals living in each household (Spearman's rank correlation coefficients, 0.14, 0.09 and 0.05 for the first, second and third surveys respectively; all $P > 0.05$). Of note, at the end of the 2013 transmission season, most mosquito collections performed after mid-November were unsuccessful, suggesting a village-wide reduction in mosquito abundance during this period.

Table 1. Study surveys

	First Survey	Second Survey	Third Survey
Start Date	October, 2013	June, 2014	September, 2014
Number of sampling days	54	20	20
Timing	End of transmission season	Start of transmission season	Peak of transmission season
Number of households	35*	20	20
Number of participants	127	81	77
Age categories**	<i>N (%)</i>	<i>N (%)</i>	<i>N (%)</i>
< 5 years	20 (15.9)	12 (14.8)	12 (15.6)
5 - 15 years	62 (49.2)	39 (48.2)	37 (48.0)
> 15 years	44 (34.9)	30 (37.0)	28 (36.4)
Gender			
Male	41 (32.5)	22 (27.2)	21 (27.3)
Female	85 (67.5)	59 (72.8)	56 (72.7)
Prevalence of falciparum parasites	99 (79.2)	-	-
Prevalence of falciparum gametocytes	79 (64.2)	-	-
Number of bloodfed mosquitoes collected	190	325	620

*Demographic information not available for individuals living in 5/40 households (first survey only);

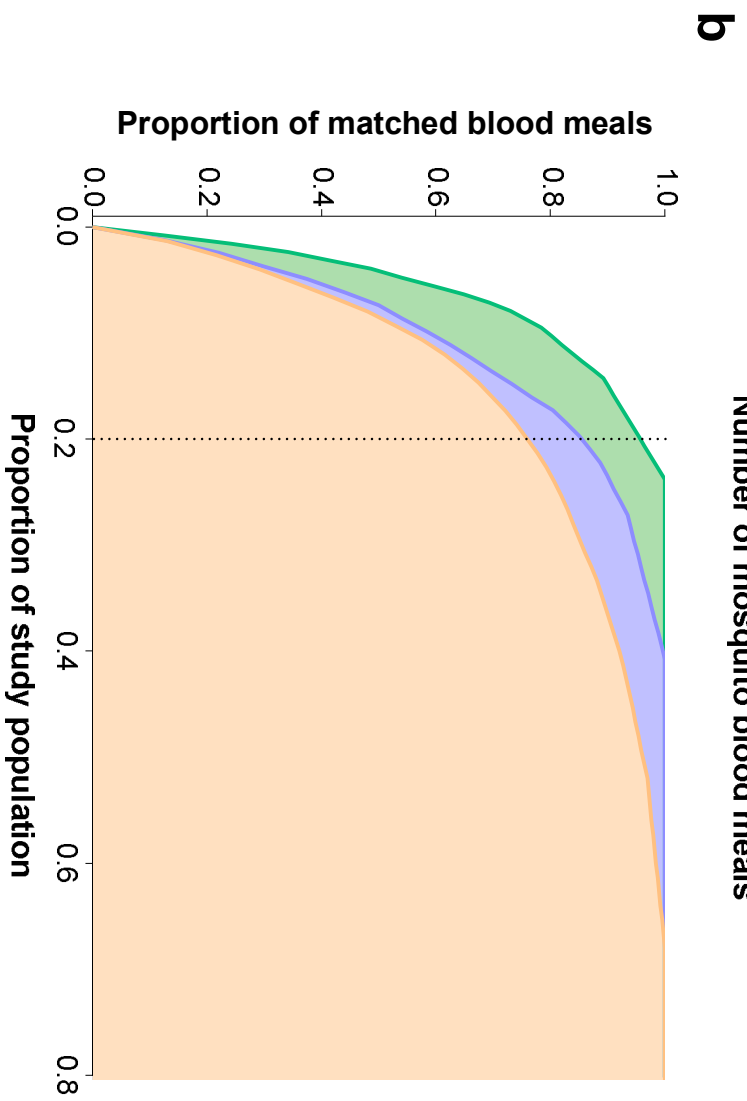
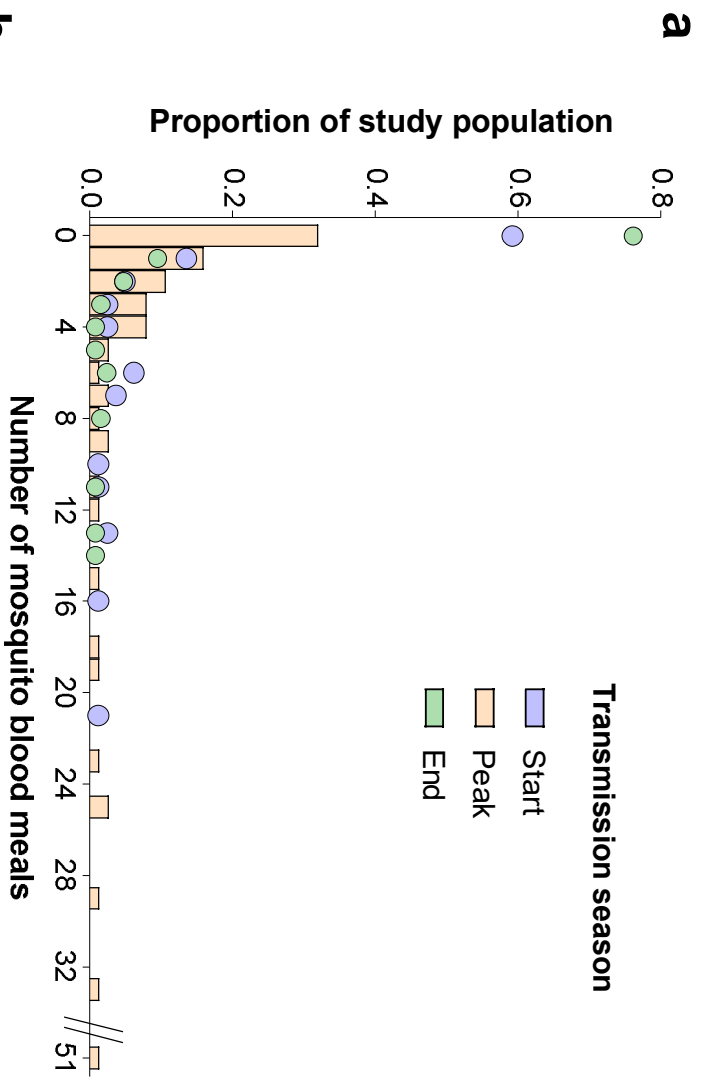
**Age at enrolment (first survey)

Heterogeneity in exposure to malaria vectors

DNA extracted from mosquito blood meals and blood samples from individuals living in study households were genotyped using a microsatellite-based assay (see *Materials and Methods*). Most (1,066/1,135, 93.9%) collected bloodfed mosquitoes had their blood meal analysed. One hundred mosquitoes did not produce discernible amplification products. Mosquito blood meals with multiple human DNA sources (N = 153), and typed blood meals that were not matched to residents of study houses (N = 139) were not included in this analysis. Estimates of mosquito exposure were based on 68.9% (666/966) of the successfully typed blood meals; this percentage was consistent across surveys (64.9, 71.7 and 69.8% at the start, peak and end of transmission season). In **Figure 1**, the distributions of the number of mosquito bites each individual received during different study surveys are presented. Approximately 20% of individuals, of all ages, provided 85.1, 76.0 and 95.5% of single mosquito blood meals at the start, peak and end of the transmission season, respectively. Throughout the study, a small number of individuals, mostly adults, were matched to considerably higher numbers of blood meals compared to the rest of the population. Conversely, 32.0 – 76.2% of study participants were not linked to bloodfed mosquitoes during the surveys, including 15/77 individuals present in all surveys who were never matched to collected fed mosquitoes. Reported bed net use and parasite and gametocyte carriage at enrolment were not significantly associated with mosquito exposure during our study (incidence rate ratio of 0.70 [95% confidence interval, CI, 0.22 – 2.20, P = 0.55] for reported bed net use versus no net use in a model adjusted for survey and of 3.42 and 0.99 [95% CI 0.50 – 23.45 and 0.32 – 3.10, P = 0.21 and 0.99] for parasite and gametocyte carriage at enrolment, respectively, in models that only included data from the first survey). In an analysis of data from all surveys, negative binomial regression with mixed effects better explained the distribution of mosquito bite counts than mixed effects Poisson models (P < 0.001), after adjustments for age, which influences *Anopheles* exposure in this population (11), survey and intra-household data correlation. In sensitivity analyses that assigned blood meals with multiple human DNA sources to the least exposed individuals in each household, the ~20% of the population with most mosquito bites were linked to at least 70% of matched blood meals (*Supporting Information Figure S1*). We also performed an analysis that assigned multiple source blood meals to study participants based on minimal genetic distances to those meals (11). This approach results in a similar

pattern: ~20% of the population received 78.6, 76.3 and 94.2% of all mosquito bites at the start, peak and end of the transmission season, respectively.

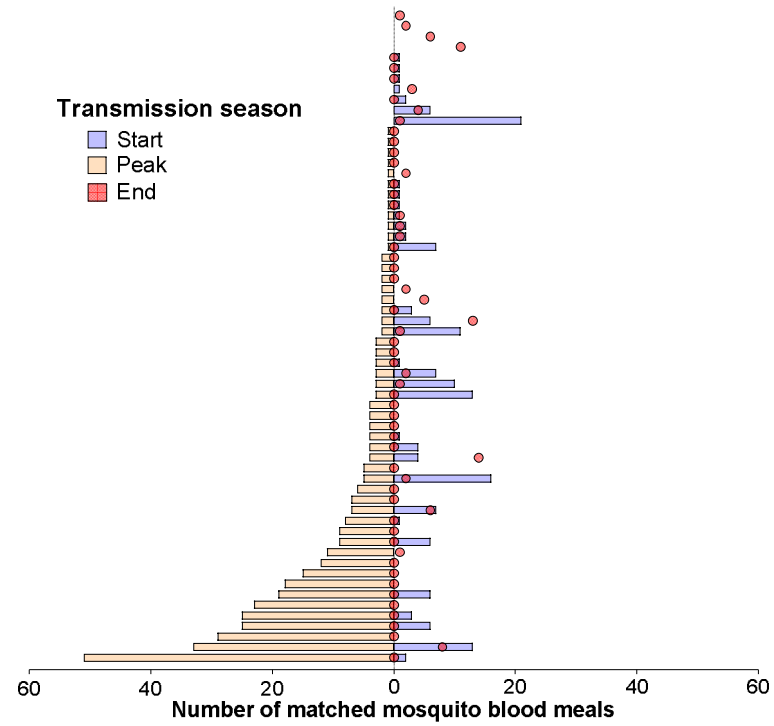
Figure 1. Inter-individual variation in exposure to *Anopheles* mosquitoes. In **a**, the frequency distributions of mosquito blood meals matched to each study participant are presented for the three different surveys. Data from the peak transmission season are presented as bars; data from the other surveys (non-zero proportions) are presented as coloured circles. In **b**, the cumulative proportion of mosquito blood meals (y-axis) matched to study participants (x-axis) sorted by number of mosquito bites received is presented for the different surveys. The points where the dotted vertical line intersects the three curves correspond to the highest proportions of mosquito blood meals linked to 20% of the study population. At the peak of the transmission season, six individuals were absent or only present during one collection day and were not included in this graph. Only singly matched bloodfed mosquitoes linked to individuals living in the same household where they were collected are included in this figure.



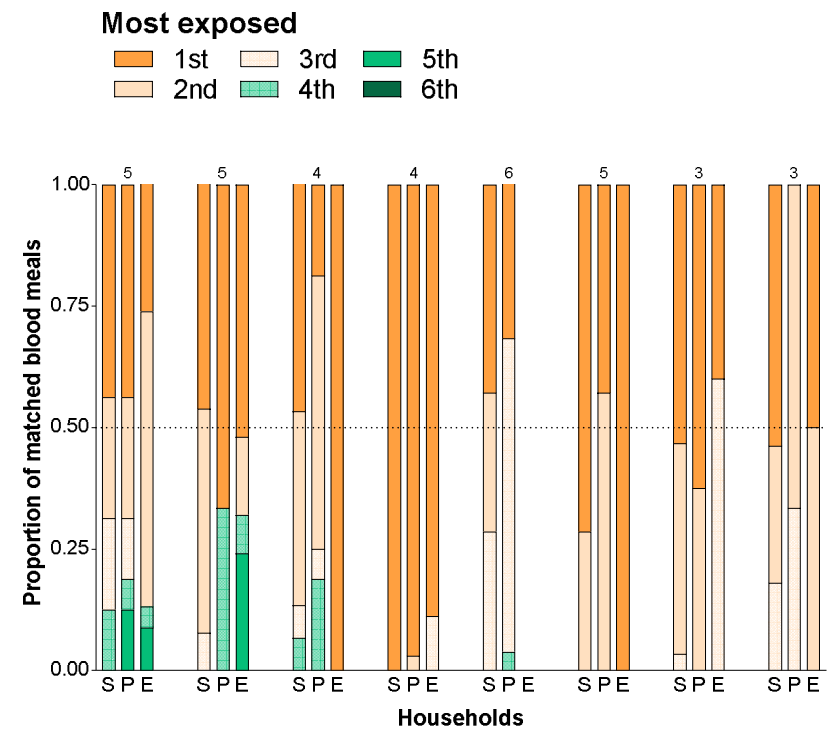
For individuals who participated in all surveys, there were positive correlations between numbers of matched mosquitoes 1) at the start and peak of transmission season (Spearman's rank correlation coefficient 0.24, $P=0.04$) and 2) at the start and end of transmission season (Spearman's rank correlation coefficient 0.34, $P=0.002$), suggesting some consistency in preferential biting. However, some individuals with highest numbers of matched blood meals at the peak of the transmission season received few or no mosquito bites in other surveys (**Figure 2a**).

Figure 2. Temporal (**a**) and within-household (**b**) variation in exposure to malaria vectors. In **a**, individuals were ranked (y-axis) according to the number of matched mosquito blood meals at the peak of the transmission season. The left and right, symmetrical, x-axes represent the number of mosquito bites each individual received at the peak (orange bars), and at the start (blue bars) and end (red circles) of the transmission season respectively. Only individuals present during at least 3 collection days per survey in all surveys and matched to at least one blood meal (N=62) are included in this panel. In **b**, each group of three columns corresponds to a different survey (S, Start of transmission season; P, Peak of transmission season; E, End of transmission season) for a select number of households. Individuals in the same household are denoted by different colours, which are consistent in the different surveys. The proportions of matched blood meals linked to each individual by household and survey are on the y-axis; only the 8 households with at least 5 matched mosquitoes at the start of the transmission season and three or more study participants are shown. The numbers of individuals living in the households included in panel **b** are shown above the columns.

a



b



Within-household heterogeneity in mosquito exposure was observed (**Figure 2b**): considering data from houses with at least three study participants and five matched mosquitoes in single surveys, in 4/8, 14/15 and 7/7 households at the start, peak and end of the transmission season respectively, the most exposed individual was the source of at least 50% of matched mosquito blood meals. While individuals with high numbers of matched blood meals often lived in households with high total numbers of matched mosquitoes (*Supporting Information Figure S2*), in all houses included in this study there were individuals with relatively low mosquito exposure.

Anopheles species-specific feeding choices

Mosquitoes collected during the study were genotyped for species identification. At the start of the transmission season, *Anopheles coluzzii* represented 44.7% (142/318) of all bloodfed mosquitoes, while at the peak and end of the transmission season most bloodfed mosquitoes were *Anopheles gambiae sensu stricto* (74.0 [450/608] and 53.7% [102/190], respectively). In all surveys, *A. coluzzii* mosquitoes had higher percentages of blood meals from multiple human sources (20.6, 22.0 and 18.9% at the start, peak and end of the transmission season, respectively) compared to *A. gambiae s. s.* mosquitoes (11.9, 16.1 and 10.3%). Species-specific distributions of blood meals singly matched to study participants are presented in **Figure S3** (*Supporting Information*) and suggest that heterogeneity in exposure to anopheline mosquitoes occurs irrespective of vector species. Of note, negative binomial models better explained the distribution of *Anopheles* species-specific mosquito bites compared to Poisson models, providing evidence of overdispersion that is not explained by covariates. In these models, the conditional overdispersion parameter was significantly different from zero: 1.9 (95% CI, 1.2 – 3.2) and 2.5 (95% CI, 1.8 – 3.6) for models of *A. coluzzii* and *A. gambiae s. s.* mosquito bites, respectively. Additionally, rates at which individuals were bitten by mosquitoes of different species were positively associated (*Supporting Information Figure S4*) (all $P < 0.001$ in mixed effects negative binomial models that included number of *A. coluzzii* or *A. gambiae s. s.* matched blood meals, age and survey as covariates).

Exposure to infected mosquito bites

The prevalence of malaria parasites in bloodfed mosquitoes identified via PCR of head and thorax was higher at the end versus start and peak of the transmission season (23.4,

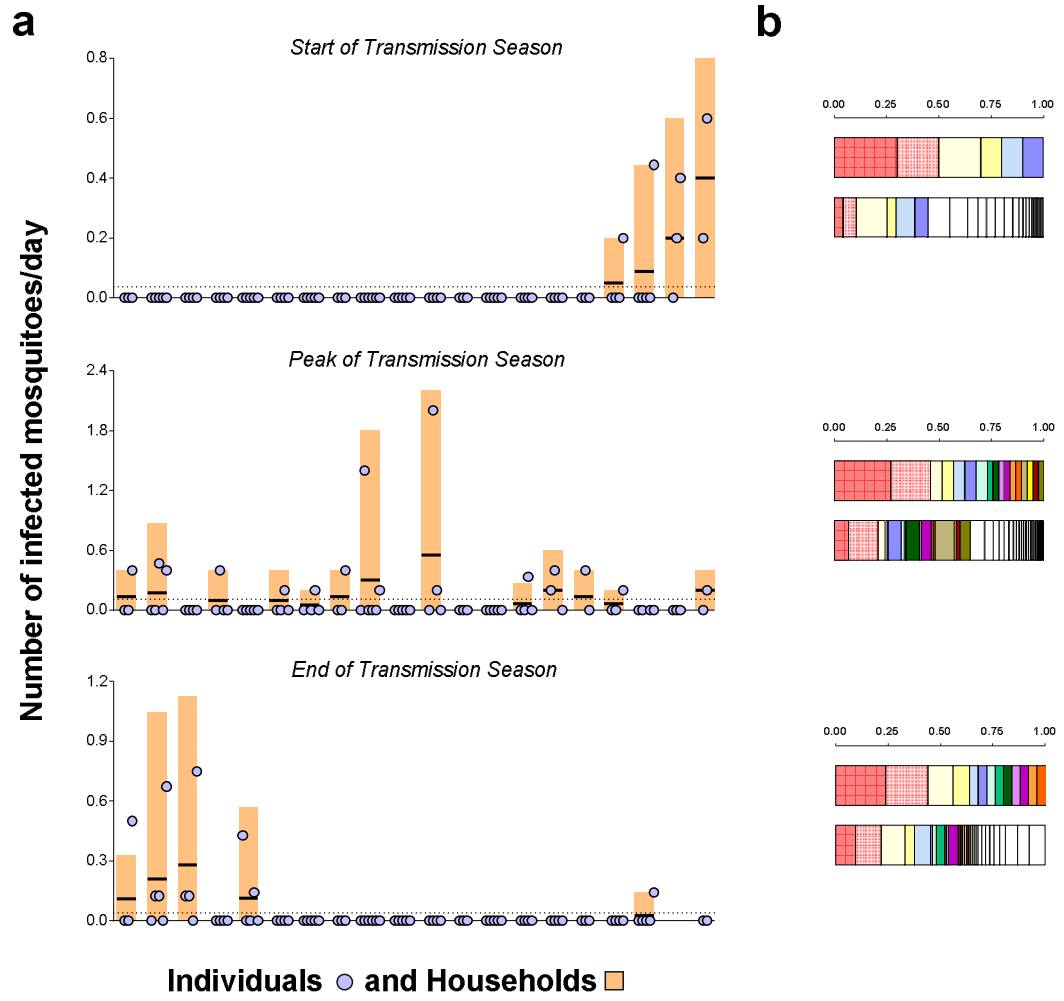
4.9 and 8.0%, respectively), and slightly higher in singly-matched mosquitoes compared to mosquitoes with multiple meal sources (**Table 2**). *A. coluzzii* mosquitoes were less often infected compared to *A. gambiae* s. s. (odds ratio 0.41 95% CI, 0.22 – 0.76 in a model that adjusted for timing of survey). Individual- and household-level frequencies of exposure to infected mosquitoes are presented in **Figure 3a**. Only 7.7, 23.6 and 10.5% of study subjects were linked to one or more potentially infectious mosquito meals at the start, peak and end of transmission season, respectively. In each survey, the two individuals with the highest numbers of matched meals from infected mosquitoes experienced 44 – 50% of all exposure to infected mosquitoes. As expected, there was a positive association between the number of potentially infective mosquito bites an individual received and the total number of matched meals regardless of mosquito parasitological status (**Figure 3b**, $P < 0.001$ in a mixed effects negative binomial model that had the total number of matched blood meals as a covariate and only included individuals with at least one matched meal). Conditional on the total number of bites, there was evidence of household-level clustering of infectious bites (intra-class [household] correlation 0.19, 95% CI 0.08 – 0.38, $P < 0.001$ in a mixed effects logistic model that included singly matched mosquitoes); however, there was no evidence of individual-level clustering after adjustment for household-related variability ($P = 0.50$).

Table 2. Prevalence of mosquito infection, determined by PCR performed using mosquitoes' head-thoraces, by blood meal source (A) and mosquito species (B), and results of mixed effects logistic model on mosquito infection status (C).

A			
Prevalence of mosquito infection by blood meal source			
	<i>Singly-matched, % (n/N)</i>	<i>Non-matched % (n/N)</i>	<i>Multiple human sources, % (n/N)</i>
Timing (Transmission Season)			
<i>Start</i>	6.2 (10/160)	4.5 (2/44)	3.3 (3/91)
<i>Peak</i>	9.9 (37/375)	3.3 (2/61)	0 (0/41)
<i>End</i>	23.1 (25/108)	25.0 (7/28)	21.1 (4/19)
B			
Prevalence of mosquito infection by mosquito species*			
	<i>A. gambiae s. s., % (n/N)</i>	<i>A. coluzzii, % (n/N)</i>	<i>A. arabiensis, % (n/N)</i>
Timing (Transmission Season)			
<i>Start</i>	4.9 (5/101)	2.5 (3/118)	9.7 (3/31)
<i>Peak</i>	9.3 (36/386)	3.8 (4/106)	12.5 (1/8)
<i>End</i>	31.4 (27/86)	15.4 (8/52)	0 (0/11)
C			
Mixed effects logistic model on infection status*			
	Odds ratio (95% CI)	P-value	
Mosquito species			
<i>A. gambiae s. s.</i>	Reference		
<i>A. coluzzii</i>	0.41 (0.22 – 0.76)	0.005	
<i>A. arabiensis</i>	0.60 (0.19 – 1.84)	0.37	
Timing (Transmission Season)			
<i>End</i>	Reference		
<i>Start</i>	0.20 (0.09 – 0.45)	<0.001	
<i>Peak</i>	0.22 (0.11 – 0.44)	<0.001	

*Only mosquitoes with amplified human DNA were included in these calculations

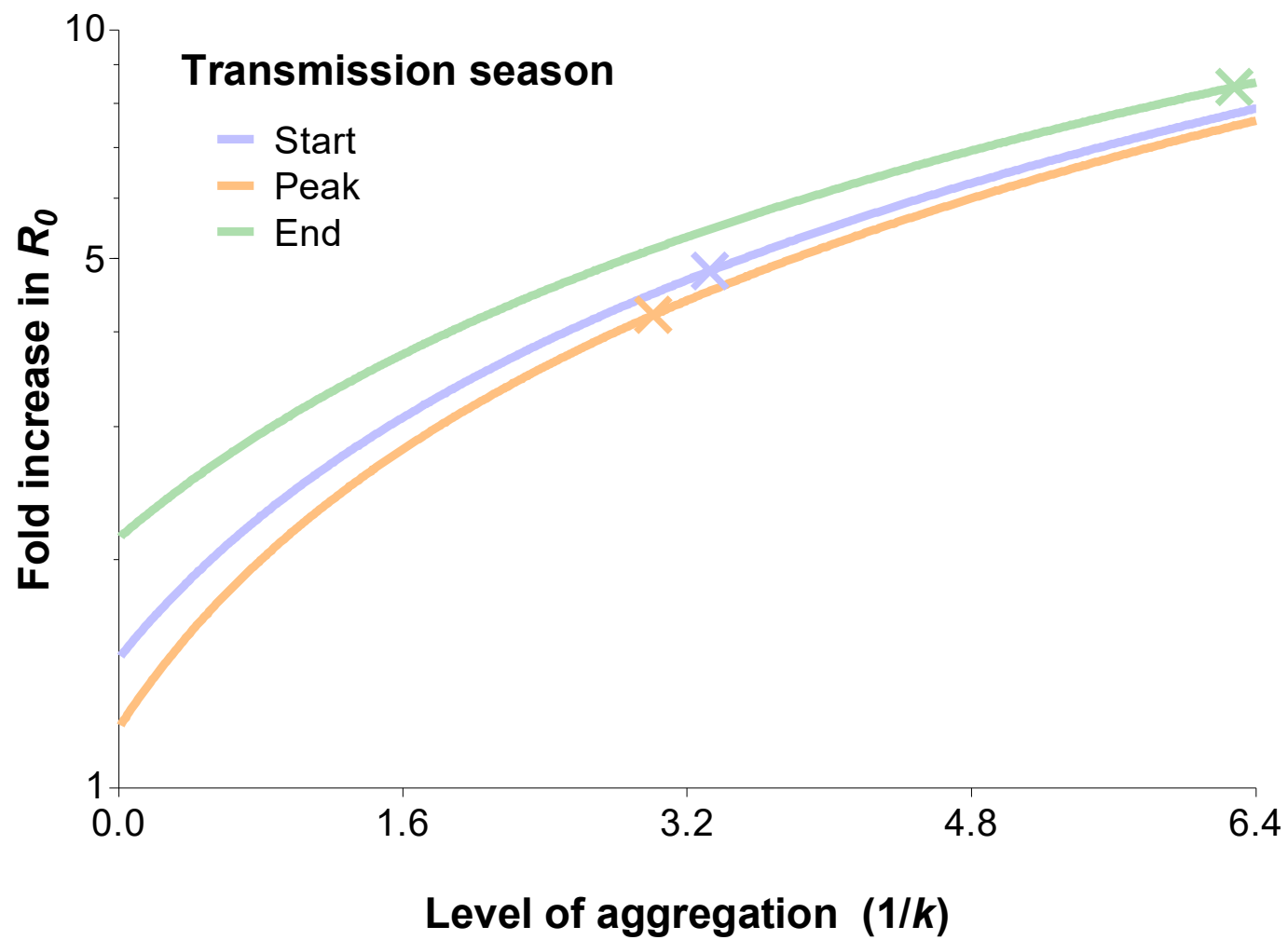
Figure 3. Rates of exposure to potentially infective mosquito bites (i.e., bites from mosquitoes with malaria parasites detected in their head-thoraces) (**a**) and their association with total mosquito exposure (**b**). In **a**, blue circles represent study participants; individuals living in the same study house are presented together (orange bars). Y-axes show *i*) the numbers of falciparum-positive mosquito bites per individual per day (blue circles), *ii*) the total number of falciparum-positive fed mosquitoes collected in each house per collection day (orange bars), and *iii*) the number of falciparum-positive fed mosquitoes collected in each house per individual resident in the house per collection day (black horizontal lines in orange bars); y-axes' limits vary to improve visualization. Horizontal dotted lines represent the average of *iii* over all houses. Only the 20 houses included in all three surveys are represented in this figure. In the three graphs, houses were ordered according to the number of infected fed mosquitoes collected at the start of the transmission season. In **b**, in each pair of bars, each colour represents an individual: the top bar represents individuals matched to infected mosquitoes; the bottom bar, individuals matched to mosquitoes regardless of sporozoite status. Individuals who were only matched to uninfected mosquitoes are represented by white segments. The horizontal axes represent proportions of blood meals. Multiple source meals are not included in panel **b**.



Effect of exposure heterogeneity on malaria R_0

The impact of biting heterogeneity on the resilience of vector-borne disease transmission to control interventions can be estimated using the methods described in (12, 13): compared to uniform biting, the basic reproduction number (R_0) of a vector-borne disease, which corresponds to the number of secondary infections generated by an infectious individual in the absence of any control and any population immunity, is inflated by the factor $(1 + \alpha)$, where α is the squared coefficient of variation of the human biting rate. **Figure 4** illustrates the impact that more extreme heterogeneity can have on the calculated R_0 of malaria: R_0 is increased by a factor of 4.8, 4.2 and 8.4 in settings where mosquito exposure and its variability are similar to those observed at the start, peak and end of the transmission season in our study area. R_0 is high even when the skew is reduced by the conservative sensitivity analysis that allocates multiply fed mosquitoes' meals to the least exposed individuals, yielding R_0 increases by a factor of 3.3, 3.2 and 5.7 at the start, peak and end of the transmission season, respectively.

Figure 4. The relationship between the fold increase in R_0 and the level of aggregation in mosquito bites. This relation depends on the mean mosquito biting rate (see below), and the different curves represent the shape of this effect for different mosquito exposure levels: blue, orange and green lines correspond to settings where host-vector contact rates (mean numbers of matched mosquito bites) are similar to those observed at the start, peak and end of the transmission season, respectively. Aggregation level increases along with the inverse of the aggregation parameter k from the negative binomial distribution, where k can be calculated as the squared mean number of mosquito bites (μ^2) divided by the variance in bite number (σ^2) minus the mean, i.e. $k = \frac{\mu^2}{(\sigma^2 - \mu)}$. R_0 is inflated by factor $(1 + \alpha)$ whereby $\alpha = \left(\frac{\sigma}{\mu}\right)^2$; in other words, α is the squared coefficient of variation (14). In the special case whereby bites are perfectly homogenous, the coefficient of variation is zero, and the standard formulation for R_0 is regained (15, 16). The fold increases in R_0 for the levels of aggregation observed in our surveys are represented by the coloured crossed-lines.



Discussion

In this study, we quantified natural exposure to *Anopheles* mosquitoes using blood meals linked to household occupants. We observed significant differences in the numbers of bloodfed mosquitoes matched to study participants that are consistent with or even exceeding the Pareto rule, with ~20% of individuals being the source of more than 80% of all singly-matched blood meals. This heterogeneity was also apparent within-household, where the individual who received the most bites often contributed more than 50% of anopheline meals. Exposure to potentially infective mosquito bites also followed an aggregated pattern: 5.1, 13.9 and 6.5% of the population experienced 80% of parasite inoculations at the start, peak and end of the transmission season, respectively. Taken together, our observations indicate that relatively few individuals at different timepoints disproportionately contribute to malaria transmission by being repeatedly sampled and infected by malaria vectors. These data provide further insights into the mechanisms that lead to heterogeneity in human malaria infection risk (17-20).

Several previous studies have quantified inter-individual differences in attractiveness and exposure to mosquitoes using a range of methodologies. While studies involving experimental huts (1) and olfactometers (21) demonstrated the influence of individual-level factors such as pregnancy (22) and infection status (23) on attractiveness to mosquitoes, community-wide assessments of wild-caught mosquitoes (8, 24, 25) are necessary to quantify variation in exposure to malaria vectors over larger scales. A study in The Gambia (8) in the 1980s used ABO group and haptoglobin typing to identify the sources of mosquito blood meals; the small number of variants in these markers (26) limited the selection of households. In Tanzania (24), microsatellites were used to link blood meals to humans to assess the protection afforded by bed nets against mosquitoes; a high proportion of the ~250 analysed bloodfed mosquitoes were matched to individuals sleeping in the same room where they were collected, including in the village with bed nets, and 80% of matched blood meals came from less than 20% of the population. In our study, we matched 666 blood meals to individual study participants and observed that the distribution of mosquito bites was highly overdispersed in relation to a Poisson assumption, with 76.0 – 95.5% of singly matched blood meals originating from ~20% of the study population. These results corroborate the findings of the study in Tanzania and of a different study in western

Kenya (27), where 16% of the study participants were matched to 58% of *Anopheles* blood meals. By examining mosquito exposure repeatedly in an area of pronounced seasonality, we demonstrate even more unequal exposure patterns across three timepoints in the season. Applying simple, well-established (14, 28) methods, we estimated that the observed degree of mosquito biting heterogeneity could be linked to a 3 fold or higher increase in malaria R_0 compared to a random-mixing system. This suggests that in settings with considerable variation in mosquito exposure, heterogeneity in biting is likely to be a major determinant of the coverage of vector control interventions required to reduce and interrupt transmission (14). These R_0 -focused calculations however have limitations. Firstly, although there is a positive relationship between R_0 and variability in *Anopheles* biting, in finite populations increasing aggregation in exposure does not necessarily equate to increasing community-wide infection burden, since in areas with highly heterogeneous mosquito biting a high proportion of parasite inoculations will be on already-infected individuals. Additionally, the framework used here does not account for previously observed interactions between frequency of exposure to infected mosquitoes and rates of immunity acquisition (17, 18, 29), which could influence blood stage parasite burden, and potentially production of transmission stages, in highly exposed, highly immune individuals. Development of detailed mathematical models that explicitly incorporate immunity and spatial dependencies (see next paragraph) would allow more precise estimation of the long-term influence of the observed variation in mosquito exposure on malaria transmission dynamics.

The fact that the majority of human-mosquito encounters in each survey involved only a few individuals is a consequence of both between- and within-household variation in exposure. Indeed, at the peak of the transmission season, the household with the highest number of fed mosquitoes had ~150 times more than the household with the lowest number. Household characteristics, such as construction material, number of windows, eaves as well as geographical proximity to mosquito breeding sites have all been previously associated with increased exposure to mosquitoes indoors and explain some of the between-household variation in mosquito abundance. The significant differences in vector biting between study houses also imply that malaria control programmes could target households with high mosquito densities. However, quantification of the temporal variation in household-level vector densities as well as

estimations of the scale of transmission (30), i.e. the distance range within which mosquitoes infected by a single infectious individual circulate and cause secondary human infections, would be necessary to determine the feasibility and relevance of these strategies. Data on geographical clustering of *Anopheles* exposure, malaria infection and disease might be informative to understand spatial-temporal changes: a study in Kenya (17) showed that foci of clinical malaria are unstable and that one-month surveillance data, for example, might only have predictive value of higher-than-average transmission over short periods of time. This is consistent with our observation that the ranking of households according to the number of infected mosquito bites per individual varies over time (**Figure 3**) and poses considerable challenges to sustainably targeting high exposure households for maximum community benefit.

We also found that household heterogeneity is compounded by inter-individual variation in frequency of sampling by mosquitoes for people in the same house. At the peak of the transmission season, the maximum difference in numbers of matched meals for participants in the same house ranged from 1 to 51, i.e. a difference of up to ~10 mosquito bites per day. Age and body size can partially explain differences in attractiveness (4) yet we often noted age-matched individuals in the same house with dissimilar mosquito exposure. Other individual-level characteristics such as odours (21, 31), (effective) use of protective measures or behaviour will be relevant; in our study, bed net use and gametocyte carriage were not significantly associated with the number of mosquito bites an individual received though this study was not designed to assess the influence of these factors on mosquito exposure. Of note, the age distribution of our study population (**Table 1**) might not reflect the true demography of the region. Whilst our results are in agreement with previous studies, we cannot exclude that a difference in age composition related to the fact that only houses with at least one child were included in the study may have influenced our heterogeneity estimates. We also observed that blood meals from only 8.2% of tested mosquitoes did not amplify for human DNA. Although it is possible that some of these mosquitoes fed on domestic animals, since it is unlikely that non-human biting would divert bites from specific hosts, we do not expect this to bias our individual-level estimates.

A technical limitation of this study was that our analyses only used data from ~70% of all successfully typed mosquito meals. Of those not matched, 139/292 were single blood meals from individuals not in our study houses and 153/292 had evidence of multiple blood meals. The distribution of these bites, each representing at least two host-vector encounters, is likely to be an important determinant of *Anopheles* exposure, in particular if some individuals are over-represented in these mixed meals, for example due to frequent defensive behaviour that leads to interrupted mosquito feeding and multiple probing. However, in a conservative sensitivity analysis that assigned these meals to the individuals with lowest exposure in each household, most mosquito bites were still linked to a small proportion of the population. Those mosquitoes whose blood meals did not match residents of study houses are likely to have fed in neighbouring houses or outdoors before entering study houses. The genetic profiles of these unmatched blood meals also suggest a heterogeneous feeding pattern: in the three houses with more than 10 unmatched mosquitoes, 16/41, 8/13 and 31/33 blood meals had the same profile. The only study house where a resident did not provide blood sample for matching had 8/10 unmatched meals presumably from the same human source. Concurrent collection of fed mosquitoes indoors and outdoors would help to understand how biting behaviour influences overall exposure.

Broad differences in anthropophily between mosquito species are well recognised (32) however there are fewer data (33) on whether different *Anopheles* species have different feeding preferences with regards to individual humans. In this study, we analysed blood meals in mosquitoes from three species: *A. gambiae* s. s., *A. coluzzii* and *Anopheles arabiensis*. Although seasonal differences in species abundance were evident, we observed aggregation in human biting irrespective of vector species and exposure to one species was positively associated with exposure to the others. Though we only collected endophagic mosquitoes, it suggests that where vectors are anthropophilic heterogeneity in exposure to anopheline mosquitoes is a common epidemiological phenomenon including in areas with mixed vector species. Additionally, we observed that the most numerous species in our study area, *A. coluzzii* and *A. gambiae* s. s., differed in two key parameters that influence the transmission potential of mosquitoes: the likelihood of feeding on multiple individuals and the prevalence of sporozoites. There is evidence from membrane feeding experiments performed in Senegal (34) that *A. gambiae* s. s. mosquitoes might be more susceptible

to malaria infection, although this association was not observed in another transmission study (35). Another possible explanation for the observed species-related difference in sporozoite prevalence could be variation in mosquito survival and consequently age structure. The higher frequency of falciparum infection in *A. gambiae* s.s. mosquitoes confirms, together with its relatively high abundance, the prime importance of *this species* for malaria transmission in the study setting. The contributions of the different *Anopheles* species to the mosquito infectious reservoir also depend on the number of potential parasite inoculations per mosquito-time: mosquitoes with multiple blood meals are more likely to infect more than one individual on a single night compared to mosquitoes with single-source blood meals, assuming sufficient quantities of sporozoites are inoculated during the probing of the different hosts. The higher proportion of blood meals with multiple human DNA sources suggests that *A. coluzzii* mosquitoes also contribute significantly to the incidence of infection in humans. Importantly, as species composition of local vector populations varies in the course of the rainy season (36), these differences might impact the rate of infection propagation in human populations over the course of a single transmission season as relative abundances of the different vector species change.

We also determined how the frequency of host-vector contacts might influence malaria infection risk in human populations. In our study area, there was considerable variation in household-specific exposure to sporozoite-positive mosquitoes (**Figure 3**). At the individual level, few individuals received multiple potentially infective bites while between 75 and 90% of the study participants were not matched to feeding by infected mosquitoes during the three study periods. Whilst this does not represent all infected mosquito bites these individuals receive during an entire transmission season, it does highlight the degree of heterogeneity in likely parasite inoculations and the limitations of using population- or region-wide entomological measures of transmission that do not capture this small-scale variation. These results also indicate that the use of blood meal genotyping with concurrent assessment of mosquito sporozoite carriage during epidemiological studies could improve our understanding of the heterogeneity in clinical malaria risk (37-39). Furthermore, whilst we observed a positive association between the total number of mosquito bites and the number of bites from sporozoite infected mosquitoes at the individual level, our data suggest

clustering of sporozoite-positive mosquito bites at the household level that was not related to local mosquito abundance. This phenomenon could be linked to *i*) a correlation between prevalence of infection in mosquitoes and in humans in the same households assuming limited mixing of mosquitoes and humans (40) (e.g. due to the presence of breeding sites near houses, reducing the distances travelled by mosquitoes between consecutive blood meals) or possibly to *ii*) factors which affect mosquito survival on a very local scale, since mosquito age is associated with cumulative risk of sporozoite infection (41).

In summary, although studies have assessed natural exposure to vectors of other infections, such as *Aedes* (42, 43) and *Culex* (44), only limited data are available for *Anopheles* mosquitoes (24, 27). In our field site, characterized by high malaria transmission intensity, we show significant heterogeneity both between and within households in terms of the number of mosquito blood meals and the distribution of potentially infectious mosquito feedings; these patterns are consistent with the 20/80 rule and support the design of interventions that aim to reduce transmission by targeting a small proportion of the population. A quantitative understanding of the processes leading to heterogeneity in mosquito exposure and its temporal variability would inform at which level such interventions may be targeted in different settings; this would require quantification of the relative contributions of household-level factors, differential attractiveness to mosquitoes and human behavioural factors.

Materials and Methods

Study area and mosquito collections

This study was conducted in Balonghin (health district of Saponé, Burkina Faso). The main economic activity in this area is subsistence farming and livestock keeping. Malaria transmission is seasonal, occurring between August and December following rainfall between June and September (45), and falciparum parasite prevalence is high, above 80% by *18S* qPCR, during the transmission season (11). At the time of this study, seasonal malaria chemoprophylaxis was not part of national guidelines in Burkina Faso. Indoor mosquito collections were performed at the end of the 2013 transmission season (October – December), and at the start (July 2014) and peak (September 2014) of the following wet season. Every week mosquito collections were performed in five households. Forty households with at least one household member

< 15 years of age were included in the first survey. For each household, mosquitoes were collected between 7 and 9 AM by mouth aspiration from walls and ceilings for a maximum of 15 minutes per sampling morning for seven days or until 30 bloodfed *Anopheles* mosquitoes were collected. In 2014, mosquito collections were performed in 20 of these 40 initial households over 10 days (5 days in July, and 5 in September). Bloodfed mosquitoes had their head-thoraces stored for speciation and malaria infection assessment by PCR (see below), and their abdomens containing blood meal material squeezed onto filter paper that was stored with desiccant until DNA extraction and further analyses.

Parasite detection in humans

Finger prick blood samples were collected at enrolment and stored in RNAprotect Cell Reagent. *18S* qPCR and *Pfs25* mRNA qRT-PCR were used to detect falciparum parasites and gametocytes, respectively.

Mosquito blood meal typing

Genetic typing of blood meal samples has been described in detail elsewhere (11). Briefly, bloodfed mosquitoes' abdomens were processed using Boom extraction method (46). The Authentifiler™ PCR Amplification kit (Applied Biosystems®), with nine human microsatellite markers and one gender marker, was used to compare human DNA in blood meals and in blood samples collected from study participants. Capillary electrophoresis was used to determine DNA profiles. Mosquito blood meals with more than two alleles in at least three loci were considered to have multiple human sources.

Mosquito speciation assay and malaria parasite detection by PCR

DNA was extracted from individual head-thoraces using the DNAzol procedure (Invitrogen). Mosquito speciation was performed using a single PCR-RFLP assay as described by Fanello and colleagues (47), and nested PCR (48) was used for *Plasmodium falciparum* detection.

Statistical analysis

Stata version 14 (Stata Corporation, College Station, TX, USA) was used for statistical analysis. Demographic data were not available for five houses where no bloodfed

mosquitoes were collected; study participants living in these households are not included in the analyses presented here. We used the number of bloodfed *Anopheles* mosquitoes collected per day per household when comparing different surveys. Wilcoxon signed-rank test was used to assess within-household changes in *Anopheles* abundance. To estimate household-specific exposure to infected mosquitoes (**Figure 3**), we only considered singly matched mosquitoes and mosquitoes with blood meals that had multiple human DNA sources, and excluded from these calculations mosquitoes whose blood meals did not match house residents; for individuals with at least one matched mosquito that was not tested for sporozoite status, the number of infected bites was estimated based on total number of matched meals and proportion of tested mosquitoes that were positive. A mixed effects logistic model was used to assess the association between mosquito falciparum infection status and mosquito species; the analysis was adjusted for survey time (fixed effect), and household of collection was used as random effect. To describe the temporal variation in relative abundance of the different *Anopheles* species, data from all mosquitoes with speciation results were used. In other analyses, that assessed *Anopheles* species-specific proportions of blood meals with multiple human sources and prevalences of sporozoites, only data from mosquitoes with both blood meal and head-thorax molecular assays were included (645/666 singly matched, 151/153 multiple, and 132/139 non-matched meals).

To generate **Figure 1b** and estimate the highest proportion of singly matched mosquito meals linked to 20% of study participants, only individuals who were present in at least 3 collection days were considered. Mixed effects negative binomial models (49, 50) were used to assess overdispersion in exposure to mosquitoes, after adjustment for variability linked to age categories and survey time (fixed effects) and household-level variation (random effect). These models were also used in *Anopheles* species-specific analysis. Likelihood-ratio test compared mixed effects negative binomial models and mixed effects Poisson models.

Ethics

Ethical clearance was obtained from the London School of Hygiene & Tropical Medicine ethics committee (reference number 6447), and the ethical review committee of the Ministry of Health of Burkina Faso (2013-7-58).

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Competing interests

The authors declare that no competing interests exist.

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Supporting Information

Figure S1. Sensitivity analyses. In **Figure 1**, only singly-matched mosquitoes were included. A total of 153 mosquito blood meals were likely to have at least two human DNA sources. In this graph, two different scenarios were assumed: in Scenario 1, each mosquito blood meal with multiple sources was assigned to the two individuals with most singly-matched meals in each household; in Scenario 2, each blood meal with multiple human DNA sources was assigned to the two individuals with lowest numbers of singly-matched mosquito meals in each household. The cumulative proportion of blood meals (y-axis) matched to study participants (x-axis) sorted by number of mosquito bites received is presented for the three different surveys and two different scenarios. Only individuals present in at least 3 mosquito collection days were included in this graph.

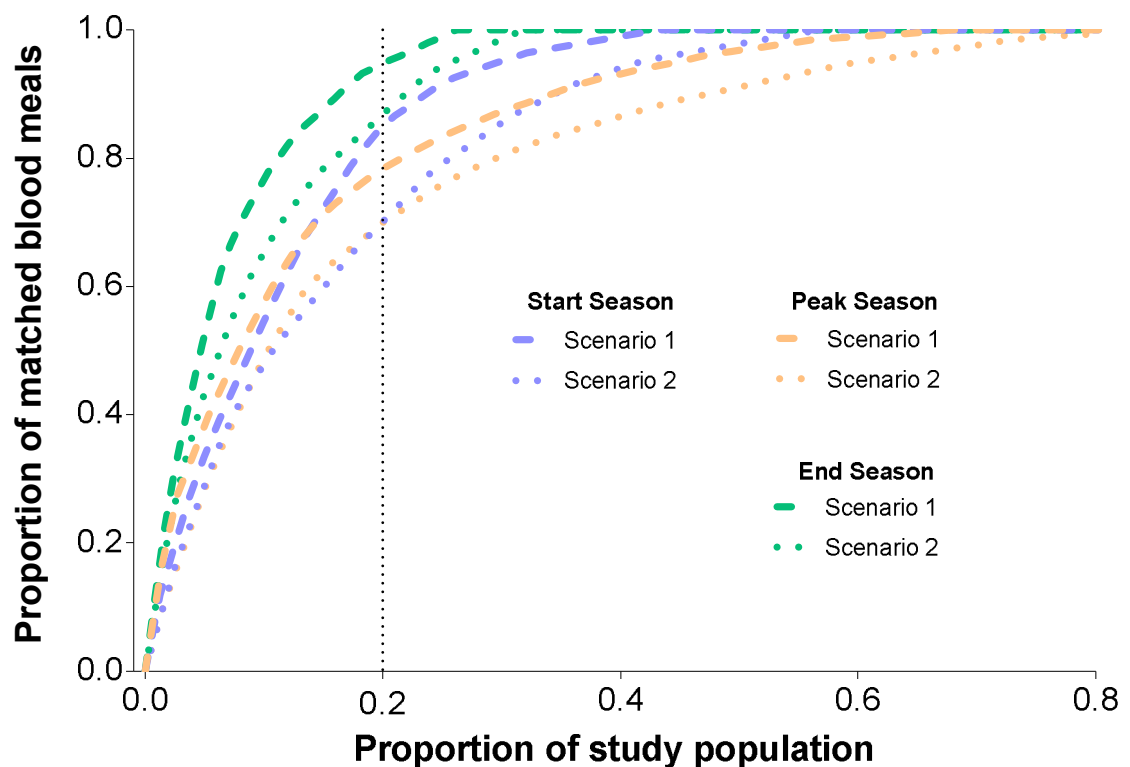


Figure S2. Relationship between household- and individual-level exposures to *Anopheles* mosquitoes. The y-axis represents the number of mosquito blood meals linked to each study participant (left column) and the total number of blood meals matched to individuals living in each study household (right column). In each panel, individuals living in the same household are represented by circles of the same colour and linked to their household. Only houses with at least 10 (start and peak of transmission season) or 5 (end of transmission season) matched mosquitoes are included in this graph; in all other houses, all individuals had 5 mosquito bites or fewer at the start or peak of the wet season, and 3 or fewer bites at the end of the transmission season.

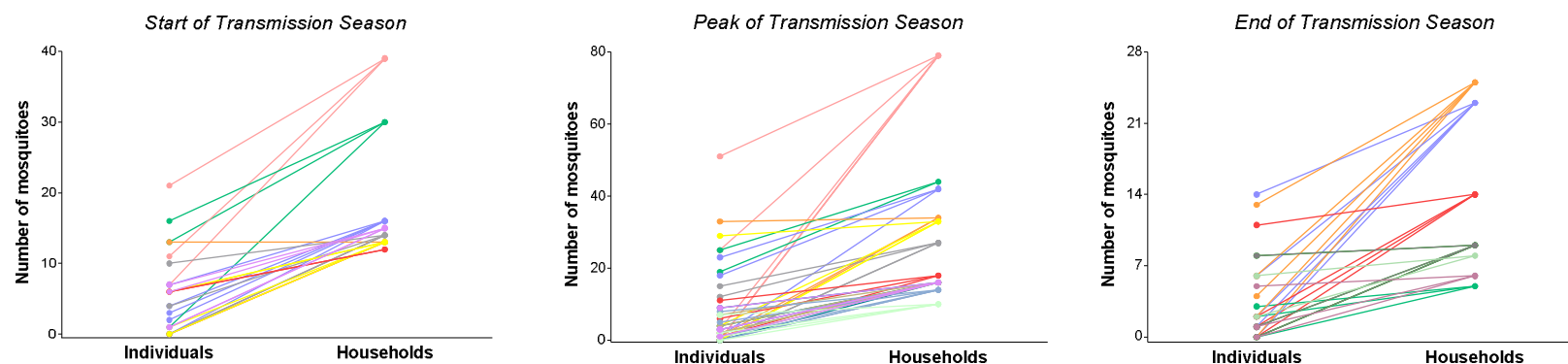


Figure S3. Cumulative proportion of *Anopheles* species-specific blood meals (y-axes) matched to study participants (x-axes) sorted by number of mosquito bites received. Only individuals present in at least 3 mosquito collection days were included in this graph. **a**, *A. gambiae* s. s.; **b**, *A. coluzzii*. Only a few (7 – 20) *A. arabiensis* mosquitoes were matched to study participants.

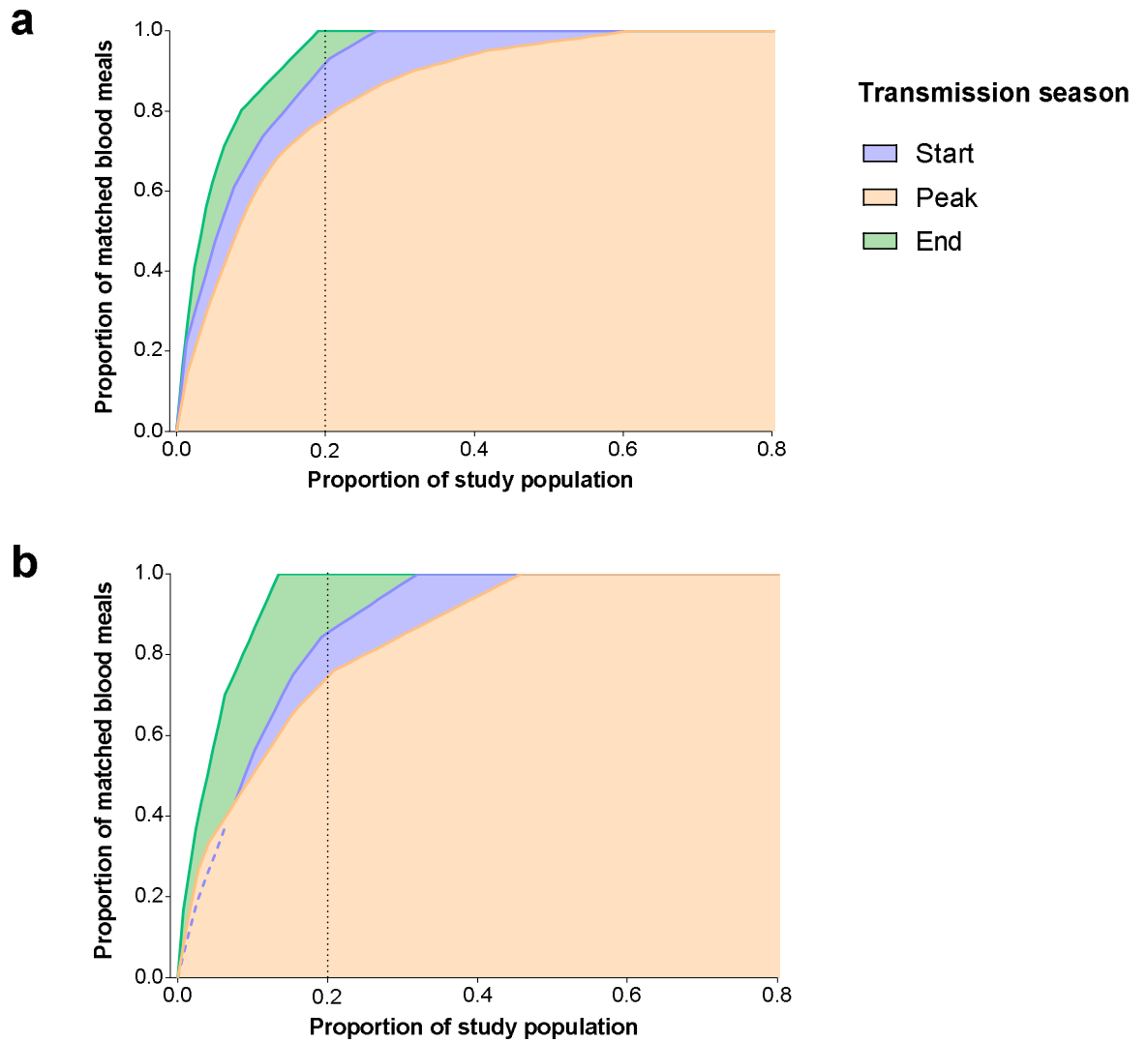
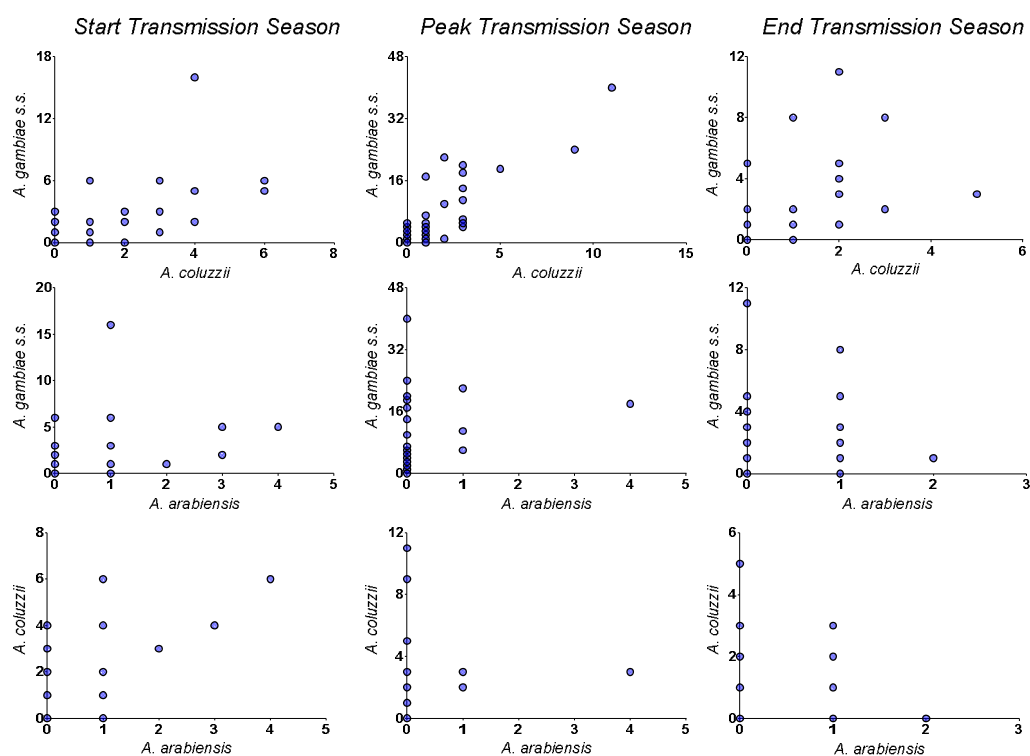


Figure S4. Correlations between vector species-specific numbers of matched blood meals. Both y- and x-axes represent the number of mosquito blood meals matched to study participants; each circle corresponds to a study participant. Axes limits vary to allow better visualisation of patterns in different surveys.



Answers to reviewers' comments

Manuscript: Variation in natural exposure to anopheles mosquitoes and its effects on malaria transmission

Reviewers' comment

SUMMARY:

This is a well-written manuscript that quantifies the uneven distribution of bites by malaria vectors within and between households in a village in Burkina Faso. With the specific exceptions noted below it appears to be a well-done study that is one of only few to document this phenomenon in the field using a large survey and capturing the detail (vector species, infected vs total bites, within and between households, demographic predictors). The phenomenon has been shown theoretically to be important, so documenting how large it is makes a significant contribution to the field, which will be interesting to anyone concerned about malaria and to others interested in the role of heterogeneity in infectious diseases. Revisions below are requested to improve the understanding of readers (including nonspecialists) of the context and precise implications of the finding.

Answer to Reviewers' comment

We would like to thank the reviewers for their detailed comments and valuable suggestions. It is gratifying that they recognise the importance of these data. We have revised the manuscript to address the points raised and to clarify the implications of our results.

Reviewers' comment

ESSENTIAL REVISIONS (these are approximately in the order they appear in the paper, but may be not exactly so as the Reviewing Editor has combined the three reviews into a single list of revisions)

1. The discussion and some of the results presentation should be modified to consider the following perspectives:

The authors cite compelling theoretical demonstrations that R_0 is massively inflated with this level of heterogeneity, compared to all behavior being average (random biting). It is not clear to me for an infection with slowly-developing immunity that R_0 is the best measure of impact. Suppose in the extreme case that all the mosquitoes exclusively bit 2 people of the 100 in a community. That would certainly make for a high R_0 and make control by say mass vaccination or mosquito control challenging (in proportion to R_0 inflation). It wouldn't however increase the burden of disease much and might decrease it, as these people would rapidly become immune, making bites on them less productive of gametocytes and further transmission (to the extent that immunity impacts transmission). Moreover (as in STI models) extreme specialization of this sort increases R_0 but not necessarily prevalence because some "nonspecialized" bloodfeeding is necessary to disperse infection into the wider community. This is an important limitation of the R_0 -focused presentation. This paper is not the place to develop the theory further, but I think emphasizing the impact on R_0 means the discursive parts of the MS should focus on the difficulty of control (which is proportional to R_0) not "transmission" or "endemicity" generally, which does not follow in a simple way.

Answer to Reviewers' comment

We agree that the fold-changes in R_0 , presented in **Figure 4**, do not necessarily reflect the true relationship between heterogeneity in exposure to mosquitoes and local human infection burden, defined as community-wide parasite prevalence or incidence of infections in uninfected individuals. We agree with the reviewers that highly heterogeneous transmission can result in an extreme scenario where the infection burden is concentrated in, or even confined to, a very small fraction of the population. In a detailed theoretical analysis (*Revisiting the basic reproductive number for malaria and its implications for malaria control*. PLoS Biology 2007), Smith and colleagues showed that in finite populations the number of different individuals whose parasites

originate from a single infected individual might decrease with increasing levels of heterogeneity, depending on the human population size. In this context, rather than being a direct measure of community-wide malaria burden, R_0 determines the coverage level of non-targeted vector control interventions required to interrupt parasite circulation.

To address this and the next comment, we have modified the following paragraph in the *Discussion* section:

*“Applying simple, well-established (24, 25) methods, ~~for assessing the influence of observed mosquito biting heterogeneity on local malaria R₀ epidemiology~~, we estimated that the observed degree of mosquito biting heterogeneity could be linked to a at least 3-times fold or higher increase in malaria R_0 than compared to it would have been in a random-mixing system. contributed significantly to the high transmission intensity in our study area. This suggests that in settings with considerable variation in mosquito exposure, heterogeneity in biting is likely to be a major determinant of the coverage of vector control interventions required to reduce and interrupt transmission (25). These R_0 -focused calculations however have limitations. Firstly, although there is a positive relationship between R_0 and variability in *Anopheles* biting, in finite populations increasing aggregation in exposure does not necessarily equate to increasing community-wide infection burden, since in areas with highly heterogeneous mosquito biting a high proportion of parasite inoculations will be on already-infected individuals. Additionally, the framework used here does not account for previously observed interactions between frequency of exposure to infected mosquitoes and rates of immunity acquisition (13, 14, 26), which could influence blood stage parasite burden and potentially the production of transmission stages in highly exposed, highly immune individuals. Development of detailed mathematical models that explicitly incorporate immunity and spatial dependencies (see next paragraph) would allow more precise estimation of the long-term influence of the observed variation in mosquito exposure on malaria transmission dynamics.”*
(Lines 299 – 303)

We have also replaced the title of the subsection “Effect of exposure heterogeneity on transmission” (*Results* section) with “Effect of exposure heterogeneity on malaria R_0 ”,

and modified its contents (some of the modifications in this paragraph relate to other comments):

*“The impact of biting heterogeneity on the resilience of vector-borne disease transmission to control interventions ~~is most transparently can be demonstrated~~ estimated using the methods described in (11, 12): compared to uniform biting, the basic reproduction number (R_0) of a vector-borne disease, which corresponds to the number of secondary infections generated by an infectious individual in the absence of any control and any population immunity, is inflated by the factor $(1 + \alpha)$, where α is the squared coefficient of variation of the human biting rate. **Figure 4** illustrates the impact that more extreme heterogeneity can have on the calculated R_0 of malaria: ~~In terms of all singly matched bloodfed mosquitoes:~~ R_0 is increased by a factor of 4.8, 4.2 and 8.4 in settings where mosquito exposure and its variability are similar to those observed at the start, peak and end of the transmission season in our study area, respectively. This intensified transmission potential remains R_0 amplification is high even when the skew is reduced by the conservative sensitivity analysis that allocates multiply fed mosquitoes’ meals to the least exposed individuals, yielding R_0 increases by a factor of 3.3, 3.2 and 5.7 at the start, peak and end of the transmission season, respectively.” (Lines 244 – 256)*

Reviewers’ comment

Another perspective on the same issue:It might be worthwhile to state that the absolute values of R_0 , as calculated, are notional in the sense that these are the values that would pertain in the absence of any control and any population immunity. In the case of Burkina, there's plenty of both control effort and immunity, so the actual R_0 is considerably less. The important lesson is that some means for better targeting control measures might be considered. Some of these are by now anodyne -- we can't merely target children, but adults must be included in our efforts. We already know that poor housing and poverty are associated with higher prevalence of malaria. While this paper very convincingly tells us that there is individual level variability in transmission -- strongly associated with spatial factors -- we don't yet know whether the observed spatial heterogeneity in transmission is temporally stable. If it isn't, then targeting of

interventions will be hard to accomplish. But for the purposes of publication decision, this is not really of importance, as the paper presents splendid data confirming some long-held theoretical and field-based suspicions.

Answer to Reviewers' comment

We agree that absolute values of R_0 should be interpreted in the context of interventions that reduce the actual reproduction number. Because of this obvious discordance, we did not estimate local malaria R_0 , opting instead to present changes in R_0 (relative to a homogenous-biting system) estimated from the new data on biting variation.

As note in the response to the previous comment we have revised the *Discussion* to point out 1) that R_0 calculations concern scenarios where no control measures are in place in a population of immunologically naïve individuals, 2) that malaria R_0 might not be the best measure to assess the impact of biting heterogeneity on local malaria burden, 3) that R_0 amplification due to variation in mosquito exposure determines requirements for control strategies to be effective, and 4) that more complex mathematical models that account for correlations between malaria exposure and rate of immunity acquisition might inform how the distribution of mosquito bites influences different human malaria metrics.

In addition to these modifications related to R_0 , we also agree with the reviewers that characterising the temporal variation in the spatial distribution of mosquito bites is essential to determine the feasibility of spatially targeted interventions. Large population-based epidemiological studies could be informative in this respect. Analysing data on more than 1,000 homesteads in coastal Kenya, Bejon and colleagues showed that foci of clinical malaria are unstable and that one-month surveillance data, for example, might only have predictive value of future hotspots for short periods of time (*A micro-epidemiological analysis of febrile malaria in Coastal Kenya showing hotspots within hotspots*. eLife 2014). This is consistent with our observation that the ranking of houses according to the number of infected mosquito bites per individual varies over time (**Figure 3**). To this, we add that determining the spatial scale of malaria transmission is also important. The average distance between

locations where consecutive blood meals of the same mosquito take place (see Perkins et al. *Heterogeneity, Mixing, and the Spatial Scales of Mosquito-Borne Pathogen Transmission*. PLOS Computational Biology 2013 for a detailed discussion on spatial scale of malaria transmission; see McCall et al. *Evidence for memorized site-fidelity in Anopheles arabiensis*. Trans R Soc Trop Med Hyg. 2001 and Le Menach et al. *The unexpected importance of mosquito oviposition behaviour for malaria: non-productive larval habitats can be sources for malaria transmission*. Malaria Journal 2005 for two possible mechanisms influencing the scale of transmission) is likely to influence the contribution of different households to community-wide transmission, and consequently is a measure of the maximal efficacy of spatially targeted interventions.

We modified the following paragraph to emphasize the need to quantify temporal variation in the spatial distribution of mosquito bites. These changes are equally relevant to comment 7.

“The fact that the majority of human-mosquito encounters in each survey ~~in our study~~ involved only a few individuals is a consequence of both between- and within-household variation in exposure. Indeed, at the peak of the transmission season, the household with the highest number of fed mosquitoes had ~150 times more than the household with the lowest number. Household characteristics, such as construction material, number of windows, eaves as well as geographical proximity to mosquito breeding sites have all been previously associated with increased exposure to mosquitoes indoors and explain some of the between-household variation in mosquito abundance. The significant differences in vector biting between study houses also imply that malaria control programmes could target households with high mosquito densities. However, quantification of the temporal variation in household-level vector densities as well as estimations of the scale of transmission (27), i.e. the distance range within which mosquitoes infected by a single infectious individual circulate and cause secondary human infections, would be necessary to determine the feasibility and relevance of these strategies. Data on geographical clustering of Anopheles exposure, malaria infection and disease might be informative to understand spatio-temporal changes: a study in Kenya (13) showed that foci of clinical malaria are unstable and that one-month surveillance data, for example, might only have predictive value of

higher-than-average transmission over short periods of time. This is consistent with our observation that the ranking of households according to the number of infected mosquito bites per individual varies over time (Figure 3) and poses considerable challenges to sustainably targeting high exposure households for maximum community benefit.

We also found that household heterogeneity is compounded by inter-individual variation in frequency of sampling by mosquitoes for people in the same house.”
(Lines 303 – 312)

Reviewers’ comment

2. Line 166 (Fig). The reviewers had divergent opinions on the figures. Two of us (ML and RP) found this figure in particular quite confusing. Another (WH) wrote "The figures are lovely, creative, and informative, once their code is understood." It is up to the authors to consider whether greater clarity can be achieved without sacrificing information or readability. Also it would be helpful to describe Fig 2 more explicitly in the text

Answer to Reviewers’ comment

We have now modified **Figure 2** to present temporal variation in exposure in one single panel. This information is now represented in linear scale (see figure below). We have also modified the *Results* section and the *Methods* section where the calculations used to create the previous panels **a** and **b** were described.

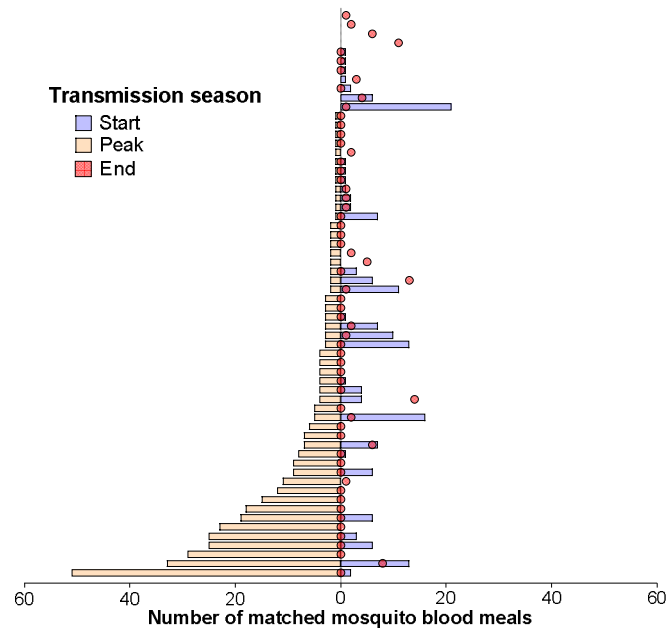
“Figure 2. Temporal (~~a-and-b~~) and within-household (**b**) variation in exposure to malaria vectors. In ~~a-and-b~~, individuals were ranked (y-axis) according to the number of matched mosquito blood meals ~~per collection day~~ at the peak of the transmission season. The left and right, symmetrical, x-axes represent the number of mosquito bites each individual received at the peak (orange bars), and at the start (blue bars) and end (red circles) of the transmission season, respectively. Only individuals present during at least 3 collection days per survey in all surveys and matched to at least one blood meal (N=62) are included in this panel. ; each green circle represents a study

~~participant and the distance between a green circle and the centre of the panel is proportional to the \log_e -transformed number of mosquito bites received per collection day (see Supporting Information for details). Each red circle corresponds to the number of singly matched blood meals at the peak (a) or end (b) of the transmission season for the individual represented by the radially aligned green circle. The dotted inner circle corresponds to no mosquito bites, and the dotted outer circle, to 5 bites per day. In **b**, each group of the three columns corresponds to a different survey (S, Start of transmission season; P, Peak of transmission season; E, End of transmission season) for a select number of households. Individuals in the same household are denoted by different colours, which are consistent in the different surveys. The proportions of matched blood meals linked to each individual by household and survey are on the y-axis; only the 8 households with at least 5 matched mosquitoes at the start of the transmission season and three or more study participants are shown. The numbers of individuals living in the households ~~included in panel e~~ are shown above the columns.”~~

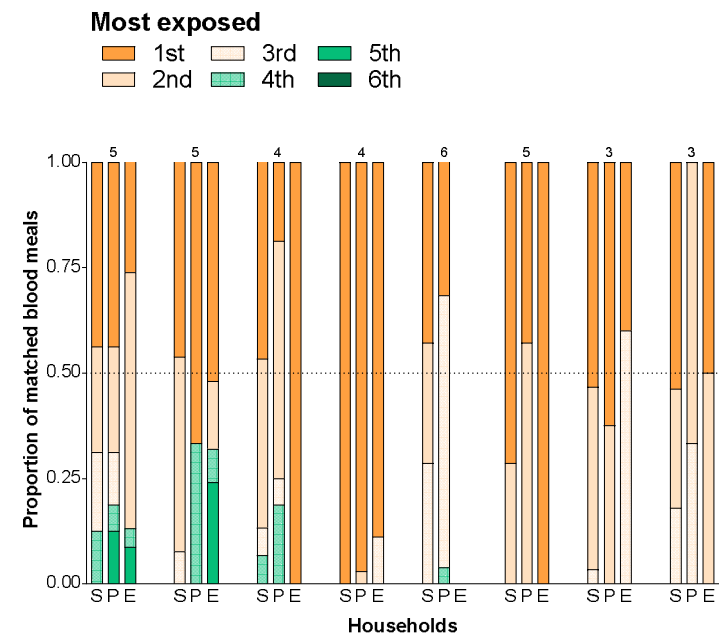
“However, some individuals with highest numbers of matched blood meals at the peak of the transmission season received few or no mosquito bites in other surveys (**Figure 2a and b**).” (Lines 151 – 153)

“Within-household heterogeneity in mosquito exposure was observed (**Figure 2b**): considering data from houses with at least three study participants and five matched mosquitoes in single surveys, in 4/8, 14/15 and 7/7 households with at least three study participants and five matched mosquitoes at the start, peak and end of the transmission season respectively, the most exposed individual was the source of at least 50% of matched mosquito blood meals.” (Lines 167 – 170)

a



b



Reviewers' comment

3, Fig 4 line 258ff. We don't understand how there are 3 different curves since $1/k$ should give a single curve. This must be because the fold increase depends on more than $1/k$, and I just don't follow the figure legend, which should provide the formula.

Answer to Reviewers' comment

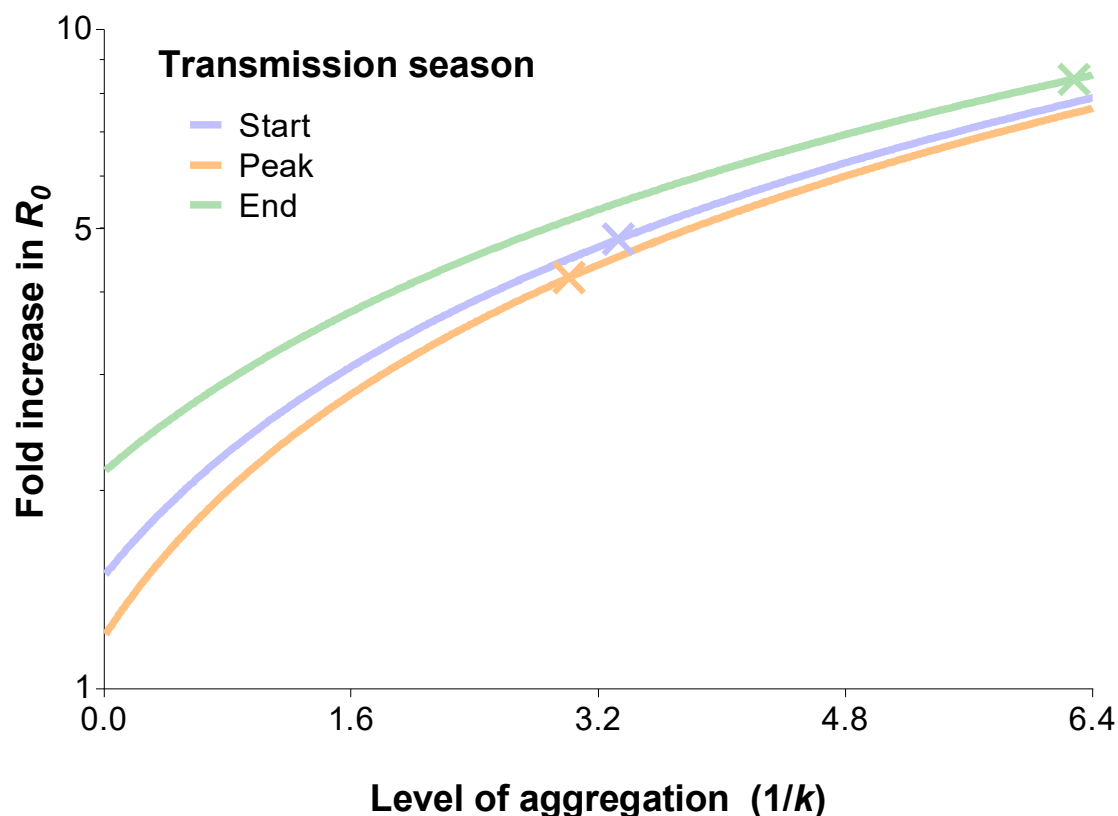
The different curves in **Figure 4** represent the effect of aggregation ($1/k$) on malaria R_0 in areas with mosquito exposure levels (i.e. average number of mosquito bites) similar to those observed in our study (i.e. start [blue], peak [orange] and end [green] of transmission season). These calculations are not intended to reflect seasonal biting variation, but rather to provide estimates of R_0 amplification in theoretical settings with vector densities and biting variation similar to those observed at different times in our study area. To clarify this, we have modified the legend of **Figure 4** and the *Results* section (see below).

In addition to addressing this comment, we wish to draw the reviewers' attention to an error that occurred in calculating the R_0 inflations. A coding error meant that the coefficient of variation was miscalculated as the variance divided by the mean bite rate when it should have been the standard deviation divided by the mean. While the biting variation recorded in the field still inflates R_0 considerably, the inflations are attenuated (now resulting in 4 to 8-fold increases in R_0 relative to homogenous mixing). We have corrected the sub-section "*Effect of exposure heterogeneity on malaria R_0* " in the *Results* section to reflect this change and apologise for not noticing this mistake sooner. These rectifications do not change the overall message of the manuscript or the large majority of the presented results. The corrected R_0 calculation resulted in changes in the last paragraph of the *Results* section and one paragraph in the *Discussion* section, the actual presentation of all entomological data is unaltered and correct.

“Figure 4. The relationship between the fold increase in R_0 and the level of aggregation in mosquito bites. This relation depends on the mean mosquito biting rate (see below), and the different curves represent the shape of this effect for different mosquito exposure levels: blue, orange and green lines correspond to settings where host-vector contact rates (mean numbers of matched mosquito bites) are similar to those observed at the start, peak and end of the transmission season, respectively for each survey, based on the mean numbers of mosquito bites recorded. Aggregation level increases along with the inverse of the aggregation parameter k from the negative binomial distribution, where k can be calculated as the squared mean number of mosquito bites (μ^2) divided by the variance in bite number (σ^2) minus the mean, i.e.

$$k = \frac{\mu^2}{(\sigma^2 - \mu)} \cdot R_0 \text{ is inflated by factor } (1+\alpha) \text{ whereby } \alpha = \left(\frac{\sigma}{\mu}\right)^2 \text{ in other words, } \alpha \text{ is the squared coefficient of variation (14).}$$

In the special case whereby bites are perfectly homogenous, the coefficient of variation is zero, and the standard formulation for R_0 is regained (15, 16). The fold increases in R_0 for the levels of aggregation observed in our surveys are represented by the coloured crossed-lines.”



“The impact of biting heterogeneity on the resilience of vector-borne disease transmission to control interventions ~~is most transparently can be demonstrated~~ estimated using the methods described in (11, 12): compared to uniform biting, the basic reproduction number (R_0) of a vector-borne disease, which corresponds to the number of secondary infections generated by an infectious individual in the absence of any control and any population immunity, is inflated by the factor $(1 + \alpha)$, where α is the squared coefficient of variation of the human biting rate. **Figure 4** illustrates the impact that more extreme heterogeneity can have on the calculated R_0 of malaria: ~~In terms of all singly matched bloodfed mosquitoes:~~ R_0 is increased by a factor of 4.8, 4.2 and 8.4 in settings where mosquito exposure and its variability are similar to those observed at the start, peak and end of the transmission season in our study area, respectively. ~~This intensified transmission potential remains~~ R_0 is high even when the skew is reduced by the conservative sensitivity analysis that allocates multiply fed mosquitoes’ meals to the least exposed individuals, yielding R_0 increases by a factor

of 3.3, 3.2 and 5.7 at the start, peak and end of the transmission season, respectively.”
(Lines 244 – 256)

Reviewers' comment

4. Line 319 and following. This result should not be presented for the first time in the Discussion. A p value with no mention of effect size or CIs is bad statistical practice. More generally, the list of factors considered as potential risk factors should be presented with univariate association measures provided (point and CI); as it is we don't know what the authors tried and only know that one association was null, but not whether it was convincingly close to null or just underpowered (narrow or wide CI respectively).

Answer to Reviewers' comment

We previously showed that age influences mosquito exposure in this area (Gonçalves et al. *Examining the human infectious reservoir for Plasmodium falciparum malaria in areas of differing transmission intensity*. Nature Communications 2017). Here, we also assessed the effect of bed net use on mosquito exposure, although as we mentioned in the *Discussion* section, the study was not designed for this. We modified the *Results* section to describe the analysis that include bed net use. Additionally, to address another comment (see “Optional major revision”), we have now included a statement on the effect of parasite carriage on mosquito biting.

“Throughout the study, a small number of individuals, mostly adults, were matched to considerably higher numbers of blood meals compared to the rest of the population. Conversely, 32.0 – 76.2% of study participants were not linked to bloodfed mosquitoes during the surveys, including 15/77 individuals present in all surveys who were never matched to collected fed mosquitoes. Reported bed net use and parasite and gametocyte carriage at enrolment were not significantly associated with mosquito exposure during our study (incidence rate ratio of 0.70 [95% confidence interval, CI, 0.22 – 2.20, P = 0.55] for reported bed net use versus no net use in a model adjusted for survey and of 3.42 and 0.99 [95% CI 0.50 – 23.45 and 0.33 – 3.10, P = 0.21 and

0.99] for parasite and gametocyte carriage at enrolment respectively in models that only included data from the first survey). In an analysis of data from all surveys, negative binomial regression with mixed effects better explained the distribution of mosquito bite counts than mixed effects Poisson models ($P < 0.001$), after adjustments for age, which influences *Anopheles* exposure in this population (11), survey and intra-household data correlation.” (Lines 117 – 124)

We have also modified the *Discussion* section:

“....; in our study, bed net use and gametocyte carriage were not significantly associated with the number of mosquito bites an individual received (~~$P=0.72$~~) though this study was not designed to assess the influence of these factors on mosquito exposure.” (Lines 318 – 320)

Reviewers' comment

5. Paragraph starting line 369 is hard to understand -- could not identify the main point.

Answer to Reviewers' comment

In this paragraph, we discuss the effect of the heterogeneity described on the distribution of potentially infectious bites from mosquitoes. As with total mosquito counts, most sporozoite-positive mosquito blood meals were matched to a small proportion of participants. An unexpected finding was that infected bites clustered at the household level after adjustment for the number of mosquitoes collected. We believe this observation is important since it might be linked to previously unrecognized epidemiological phenomena. Indeed, possible explanations for this include 1) household-level differences in mosquito survival and average age, where mosquito age is associated with cumulative risk of infection (Lines et al. *Human malaria infectiousness measured by age-specific sporozoite rates in *Anopheles gambiae* in Tanzania*. Parasitology 1991), and 2) a time-lagged correlation between prevalences of infection in mosquitoes and in humans in corresponding households.

Explanation 2 implies limited movement of mosquitoes in some areas, i.e. the distance travelled by mosquitoes between consecutive blood meals is small. This might occur for example in houses located near breeding sites, which allow oviposition after blood feeding and might increase the probability that mosquitoes feed in the same house afterwards. Another mechanism could involve feeding site-fidelity as previously described (McCall et al. *Evidence for memorized site-fidelity in Anopheles arabiensis*. Trans R Soc Trop Med Hyg. 2001).

We have improved the clarity of this paragraph. We have removed the statement that contrasted the proportion of the study population linked to potentially infective blood meals and the prevalence of infection in this population, since these two quantities are not directly comparable (we assessed exposure to infected mosquitoes during one week at the end of the transmission season, while infections in humans can last several months [Felger et al. *The Dynamics of Natural Plasmodium falciparum Infections*. PLoS ONE 2012]). We also included additional information about the clustering of infected mosquitoes at the household level.

*“We also determined how the frequency of host-vector contacts might influence malaria infection risk in human populations. In our study area, there was considerable variation in household-specific exposure to sporozoite-positive mosquitoes (**Figure 3**). At the individual level, few individuals received multiple potentially infective bites while ~~For example, at the end of the 2013 transmission season, only 8/35 study houses had infected bloodfed mosquitoes whilst 79.2% (99/125) of the study participants had falciparum parasites detected by PCR. Overall, between 75 and 90% of the study participants were not matched to feeding by infected mosquitoes during the three study periods. Whilst this does not represent all infected mosquito bites these individuals receives~~ during an entire transmission season, it does highlight the degree of heterogeneity in likely parasite inoculations and the limitations of using population- or region-wide entomological measures of transmission that do not capture this small-scale variation. These results also indicate that the use of blood meal genotyping with concurrent assessment of mosquito sporozoite carriage during epidemiological studies could improve our understanding of the heterogeneity in clinical malaria risk (33-35). Furthermore, whilst we observed a positive association between the total*

number of mosquito bites and the number of bites from sporozoite infected mosquitoes at the individual level, ~~there was no evidence that the proportion of potentially infective bites varies from individual to individual within a household. On the other hand,~~ our data also suggest clustering of sporozoite-positive mosquito bites at the household level that was not related to local mosquito abundance. This phenomenon could be linked to i) a correlation between prevalence of infection in mosquitoes and in humans in the same households ~~household-specific parasite prevalence in humans, assuming some fidelity in mosquito-host interaction, i.e. assuming limited mixing of mosquitoes and humans (31) (e.g. due to the presence of breeding sites near houses, reducing the distances travelled by mosquitoes between consecutive blood meals),~~ or possibly to ii) factors which affect mosquito survival on a very local scale, since mosquito age is associated with cumulative risk of sporozoite infection (38). ~~Whether individuals who received multiple infected bites developed infections and how different inoculations and ongoing blood stage infections interacted with each other (32) are not known. Studies that combine mosquito blood meal typing and parasitological and clinical follow-up of human populations would be informative to understand heterogeneity in clinical malaria risk (33-35).” (Lines 369—391)~~

Reviewers' comment

6. Lines 407 ff. We would like more information. I don't understand what was done. How were households chosen and individuals in them identified? Was sleeping in the household the night of the mosquito collection necessary for inclusion (if not, maybe some heterogeneity is just because someone wasn't there). What is the mosquito trapping protocol (I don't know about mouth aspiration but want more detail anyway about how long, where, what kind of traps etc)

Answer to Reviewers' comment

Households with at least one child were eligible for inclusion in the first entomological survey. Residents in study houses were enrolled and asked to provide a finger prick blood sample that was used to match mosquito blood meals to humans. In the second and third surveys, twenty houses from the first survey with no changes in the number of residents (e.g., no births or deaths) were sampled again for bloodfed mosquitoes;

priority was given to houses with highest mosquito exposure in the first survey. For the duration of catching in each survey we recorded whether each participant was present in the study house during the nights preceding mosquito collections. We used this information as exposure variable in statistical models of mosquito biting, including in the negative binomial models, and to exclude individuals with limited exposure data from some of the figures (**Figure 1**, **Figure 2** and **Figure S1**).

We have now modified the *Methods* section to give more details about mosquito collections:

*“This study was conducted in Balonghin (health district of Saponé, Burkina Faso). The main economic activity in this area is subsistence farming and livestock keeping. Malaria transmission is seasonal, occurring between August and December following rainfall between June and September (39), and falciparum parasite prevalence is high, above 80% by 18S qPCR, during the transmission season (11). At the time of this study, seasonal malaria chemoprophylaxis was not part of national guidelines in Burkina Faso. Indoor mosquito collections were performed at the end of the 2013 transmission season (October – December), and at the start (July 2014) and peak (September 2014) of the following wet season. Every week mosquito collections were performed in five households. Forty households with at least one household member ≤ 15 years of age were included in the first survey. For each household, mosquitoes were collected between 7 and 9 AM by mouth aspiration from walls and ceilings for a maximum of 15 minutes per sampling morning for seven days or until 30 bloodfed *Anopheles* mosquitoes were collected. In 2014, mosquito collections were performed in 20 of these 40 initial households over 10 days (5 days in July, and 5 in September). Bloodfed mosquitoes had their head-thoraces stored for species confirmation and malaria infection assessment by PCR (see below), and their abdomens containing blood meal material squeezed onto filter paper that was stored with desiccant until DNA extraction and further analyses.” (Lines 408 – 419)*

Reviewers' comment

7. The paper's penultimate sentence looms over this effort: "...support the design of interventions that aim to reduce transmission by a targeting a small proportion

of the population." The theoretical literature already admonishes us to do this, but your data help us to grapple with the question - should we be targeting households or individuals? I think the data clearly support the former, and would suggest that your discussion say so. Figures 3a, S2, and your regression analysis - line 214 "there was no evidence of individual level clustering after adjustment for household related variability ($p = 0.50$) all support the conclusion that the challenge to programs is to identify houses that attract the most mosquitoes, and not, thankfully, the most attractive individuals within houses. To gain this understanding, you advocate for (line 389) "studies that combine mosquito blood meal typing and parasitological and clinical follow up"; in an earlier phrase (line 386) "factors which affect mosquito survival on a very local scale" are mentioned. What about the distribution and productivity of larval habitats? In a paper about heterogeneity, this is an essential and extremely heterogeneous (spatially and temporally) fact of nature that should be considered if we are to figure out how to target the individuals living in high transmission houses. Even if you don't agree with my interpretation of your data, I think it would be worthwhile to address the 'what do we need to target? - individuals or households?' question more directly if the intent is to inform research of programmatic usefulness.

Answer to Reviewers' comment

Indeed, our results indicate that exposure to infected mosquitoes is highly variable at the household level. In particular, at the start of the transmission season, a period which may determine the malaria burden during the subsequent transmission season, only a few houses had infected mosquitoes collected. As the reviewer suggests, more effective targeting a fraction of households could have a significant impact on potential transmission. The issue of the identification of households associated with high exposure to mosquitoes is obviously key. As mentioned by the reviewer, the spatial distribution of larval breeding sites is one likely factor determining spatial heterogeneity in transmission, as it determines both abundance and feeding after oviposition, which has an effect on the scale of transmission (see Le Menach et al. *The unexpected importance of mosquito oviposition behaviour for malaria: non-productive larval habitats can be sources for malaria transmission*. Malaria Journal 2005 for a detailed discussion). However, we strongly suspect that other factors such

as household construction and adherence to existing vector control will also play key roles in this and that identification of factors will be ecotype dependent. We have now modified the following paragraph to address this comment and the related comment 1:

“The fact that the majority of human-mosquito encounters in each survey ~~in our study~~ involved only a few individuals is a consequence of both between- and within-household variation in exposure. Indeed, at the peak of the transmission season, the household with the highest number of fed mosquitoes had ~150 times more than the household with the lowest number. Household characteristics, such as construction material, number of windows, eaves as well as geographical proximity to mosquito breeding sites have all been previously associated with increased exposure to mosquitoes indoors and explain some of the between-household variation in mosquito abundance. The significant differences in vector biting between study houses also imply that malaria control programmes could target households with high mosquito densities. However, quantification of the temporal variation in household-level vector densities as well as estimations of the scale of transmission (27), i.e. the distance range within which mosquitoes infected by a single infectious individual circulate and cause secondary human infections, would be necessary to determine the feasibility and relevance of these strategies. Data on geographical clustering of Anopheles exposure, malaria infection and disease might be informative to understand spatio-temporal changes: a study in Kenya (13) showed that foci of clinical malaria are unstable and that one-month surveillance data, for example, might only have predictive value of higher-than-average transmission over short periods of time. This is consistent with our observation that the ranking of households according to the number of infected mosquito bites per individual varies over time (Figure 3) and poses considerable challenges to sustainably targeting high exposure households for maximum community benefit.”

In our opinion, targeting demographic groups should be considered in some settings. In a related analysis, we estimated that adults contribute ~50% of mosquito infections in this study area after accounting for the age structure of the population, relative infectivity and heterogeneity in exposure. One potential advantage of this approach compared to spatial targeting is the easier identification of demographic groups that

on average are more likely to contribute to transmission. Furthermore, there is considerable transmission outside hotspots of asymptomatic and clinical malaria, and by targeting for example all adults living in an endemic area transmission might be reduced in both highly exposed and less exposed households. We have included some of these ideas in the final paragraph of the *Discussion* section:

“In summary, although studies have assessed natural exposure to vectors of other infections, such as Aedes (36, 37) and Culex (38), only limited data are available for Anopheles mosquitoes (20, 23). In our field site, characterized by high malaria transmission intensity, we show significant heterogeneity both between and within households in terms of the number of mosquito blood meals and the distribution of potentially infectious mosquito feedings; these patterns are consistent with the 20/80 rule and support the design of interventions that aim to reduce transmission by targeting a small proportion of the population. Opportunities to specifically target high-exposure households depend on the operational feasibility to identify these households and the stability in exposure over time. A quantitative understanding of the processes leading to ~~the~~ heterogeneity in mosquito exposure and its temporal variability would inform at which level such interventions may be targeted household or individual, in different settings; this would require quantification of the relative contributions of household-level factors, differential attractiveness to mosquitoes and human behavioural factors.”

Reviewers' comment

8. It would be helpful to summarize key features of malaria transmission in this area: what is the prevalence of parasitemia or other measures of transmission, and what is the nature and extent of interventions in place?

Answer to Reviewers' comment

Data from a recent epidemiological study showed that the prevalence of falciparum parasites during malaria transmission season was 83.8% by molecular methods, which is similar to the prevalence of 79.2% during the first mosquito collection survey. While seasonal chemoprophylaxis was not recommended in Burkina Faso at the time of this

study, reported bed net use was high (90.0%, 87.1% and 88.6% for children < 5 years, schoolchildren and adults, as we previously described [Gonçalves et al. *Examining the human infectious reservoir for Plasmodium falciparum malaria in areas of differing transmission intensity*. Nature Communications 2017]). We now mention this information in the *Methods* and *Results* sections.

“This study was conducted in Balonghin (health district of Saponé, Burkina Faso). The main economic activity in this area is subsistence farming and livestock keeping. Malaria transmission is seasonal, occurring between August and December following rainfall between June and September (39), and falciparum parasite prevalence is high, above 80% by 18S qPCR, during the transmission season (11). At the time of this study, seasonal malaria chemoprophylaxis was not part of national guidelines in Burkina Faso. Indoor mosquito collections were performed at the end of the 2013 transmission season (October – December), and at the start (July 2014) and peak (September 2014) of the following wet season. Every week mosquito collections were performed in five households. Forty households with at least one household member < 15 years of age were included in the first survey. For each household, mosquitoes were collected between 7 to 9 AM by mouth aspiration from walls and ceilings for a maximum of 15 minutes per sampling morning for seven days or until 30 bloodfed Anopheles mosquitoes were collected. In 2014, mosquito collections were performed in 20 of these 40 initial households over 10 days (5 days in July, and 5 in September). Bloodfed mosquitoes had their head-thoraces stored for speciation and malaria infection assessment by PCR (see below), and their abdomens containing blood meal material squeezed onto filter paper that was stored with desiccant until DNA extraction and further analyses.” (Lines 408 – 419)

“We performed indoor resting collections of anopheline mosquitoes in an area with seasonal malaria transmission in Burkina Faso. Thirty-five households were included in this analysis. The median number of individuals living in each study household was 3 (range, 2 – 5). Reported bed net use among the study participants was high (111/126, 88.1%).” (Lines 81 – 83)

OPTIONAL MAJOR REVISION

Reviewers' comment

The most surprising absence, given that people were bled for human genotyping and given that LaCroix et al. (cited ref 19) showed an increased attractiveness of parasite infected people to mosquitoes, is that there is no information given on the parasite prevalence in the population at an individual or even general level (how much malaria is there and when). The mosquitoes were examined for parasite sporozoite stages, but if the bloodmeals were squeezed out for typing and the abdomens remained, then surely the mosquito bloodmeal could have been checked for parasites.

Answer to Reviewers' comment

In response to the reviewer comment, we have now included parasitology data for the first season when samples were available for this purpose. We tested these human samples, stored in RNeasy Protect cell reagent, for parasite and gametocyte detection using *18S* qPCR and *Pfs25* qRT-PCR and results are now included in the *Results* section and in **Table 1** (see below). We observed high parasite and gametocyte prevalence in human samples and, potentially because of this, no association between mosquito exposure and parasite carriage in humans. We do agree that this is an interesting phenomenon and highly valuable to explore in the context of natural mosquito exposure. Given the estimated effect sizes (~2-fold higher mosquito exposure as a consequence of gametocyte carriage (Busula *et al.* J Infect 2017; Lacroix *et al.* PLoS Biol 2005) and body surface/age (up to 21-fold higher exposure, Gonçalves *et al.* *Examining the human infectious reservoir for Plasmodium falciparum malaria in areas of differing transmission intensity.* Nature Communications 2017) it may be challenging to reliably detect and quantify an impact of human parasite status on mosquito exposure in natural settings.

“We performed indoor resting collections of anopheline mosquitoes in an area with seasonal malaria transmission in Burkina Faso. Thirty-five households were included in this analysis. The median number of individuals living in each study household was

3 (range, 2 – 5). Reported bed net use among the study participants was high (111/126, 88.1%). At enrolment (October – December 2013), most (79.2%) individuals were parasite-positive by 18S qPCR; in 21/35 houses, all individuals were infected. We collected 325, 620 and 190 bloodfed Anopheles mosquitoes at the start (2014), peak (2014) and end (2013) of the transmission season, respectively (Table 1).”

“Throughout the study, a small number of individuals, mostly adults, were matched to considerably higher numbers of blood meals compared to the rest of the population. Conversely, 32.0 – 76.2% of study participants were not linked to bloodfed mosquitoes during the surveys, including 15/77 individuals present in all surveys who were never matched to collected fed mosquitoes. Reported bed net use and parasite and gametocyte carriage at enrolment were not significantly associated with mosquito exposure during our study (incidence rate ratio of 0.70 [95% confidence interval, CI, 0.22 – 2.20, P = 0.55] for reported bed net use versus no net use in a model adjusted for survey and of 3.42 and 0.99 [95% CI 0.50 – 23.45 and 0.33 – 3.10, P = 0.21 and 0.99] for parasite and gametocyte carriage at enrolment respectively in models that only included data from the first survey). In an analysis of data from all surveys, negative binomial regression with mixed effects better explained the distribution of mosquito bite counts than mixed effects Poisson models ($P < 0.001$), after adjustments for age, which influences Anopheles exposure in this population (11), survey and intra-household data correlation.” (Lines 117 – 124)

“....; in our study, bed net use and gametocyte carriage were not significantly associated with the number of mosquito bites an individual received (~~$P = 0.72$~~) though this study was not designed to assess the influence of these factors on mosquito exposure.” (Lines 318 – 320)

“Parasite detection in humans

Finger prick blood samples were collected at enrolment and stored in RNeasy Protect Cell Reagent. 18S qPCR and Pfs25 mRNA qRT-PCR were used to detect falciparum parasites, and gametocytes, respectively.” (new sub-section in the Methods section)

“Table 1. Study surveys

	First Survey	Second Survey	Third Survey
Start Date	October, 2013	June, 2014	September, 2014
Number of sampling days	54	20	20
Timing	End of transmission season	Start of transmission season	Peak of transmission season
Number of households	35*	20	20
Number of participants	127	81	77
Age categories**	<i>N (%)</i>	<i>N (%)</i>	<i>N (%)</i>
< 5 years	20 (15.9)	12 (14.8)	12 (15.6)
5 - 15 years	62 (49.2)	39 (48.2)	37 (48.0)
> 15 years	44 (34.9)	30 (37.0)	28 (36.4)
Gender			
Male	41 (32.5)	22 (27.2)	21 (27.3)
Female	85 (67.5)	59 (72.8)	56 (72.7)
<u>Prevalence of falciparum parasites</u>	<u>99 (79.2)</u>	=	=
<u>Prevalence of falciparum gametocytes</u>	<u>79 (64.2)</u>	=	=
Number of bloodfed mosquitoes collected	190	325	620

*Demographic information not available for individuals living in 5/40 households (first survey only); **Age at enrolment (first survey)

”

MINOR REVISIONS

Reviewers' comment

1. Line 68: I don't think 9,10 focus on endemicity levels but rather on R0/growth of an epidemic in a naive population. Havent reread them but this wording should be checked for accuracy

Answer to Reviewers' comment

We have now corrected the sentence in line 68, and merged this paragraph with the following paragraph. Indeed, while Woolhouse and colleagues analysed data on a wide range of infectious agents, including some which are commonly associated with endemic transmission, such as schistosomiasis and leishmaniasis, all calculations involved R_0 , i.e. spread of these infections in naïve populations (*Heterogeneities in the transmission of infectious agents: implications for the design of control programs. PNAS 1997*).

*“The multifactorial nature of mosquito exposure in malaria endemic areas indicates that, while experimental and quasi-experimental, e.g. involving modified tents and huts, entomological studies are valuable, they will not accurately capture inter-individual variation in actual exposure. Identifying transmission heterogeneities, especially extreme heterogeneities, is however critical to better inform infectious disease epidemiology. ~~It is not unusual for a small proportion of individuals to contribute disproportionately to transmission, and well-established theory has elucidated how even small numbers of super-spreaders can trigger large outbreaks or perpetuate high levels of endemicity~~ their implications for pathogen spread and control measures (9, 10). Here, we describe the variability in natural exposure to malaria vectors by linking, through DNA fingerprinting, blood meals of wild-caught mosquitoes to humans living in the households where they were collected. Previously we have shown that these mosquitoes fed more often on adults (11). We now extend this analysis to assess the degree of heterogeneity in the distribution of mosquito bites in the population at different times during the transmission season. We also present the frequency of *Anopheles* species-specific mosquito bites, and potential parasite inoculations (i.e., sporozoite-positive mosquito bites) per individual.” (Lines 61 – 77)*

Reviewers’ comment

2. Line 258 ff. Is the inflation relative to uniform biting (everyone gets the same number of bites) or Poisson biting (more realistic)? I thin it is relative to Poisson, since in this situation the dispersion parameter would be infinite and the inflation would be 0. But I think the null is inflation of 1, so I am confused.

Answer to Reviewers’ comment

In our analysis, we used a formula developed by Dye & Hasibeder (*Population dynamics of mosquito-borne disease: effects of flies which bite some people more frequently than others*. Trans R Soc Trop Med Hyg. 1986), where R_0 amplification due to variable exposure to mosquitoes is estimated and compared to the theoretical situation of uniform biting. A similar approach was adopted by Woolhouse and colleagues (see comment above). In other words, the null is achieved when the coefficient of variation equals zero and R_0 reverts to the standard (homogenous mixing) assumption.

Reviewers' comment

3. Line 356 please elaborate on these differences and why you found them interesting

Answer to Reviewers' comment

In general, malaria vector species occur in different combinations and this influences vectorial capacity of any transmission system. This natural variation in species composition of vector populations together with the fact that different species are affected by control measures differently highlights the importance of understanding the factors influencing vector species-specific contribution to malaria transmission. In this study, bloodfed *A. gambiae* s. s. mosquitoes were more likely to carry sporozoites, and *A. coluzzii* mosquitoes had a higher percentage of blood meals with multiple human sources, which implies blood feeding on at least two individuals in a single night. The relevance of these results lies in their influence on the contribution of different mosquito species to transmission. Whilst the number of mosquito bites from different vector species depends primarily on their abundance and degree of anthropophily, the non-negligible frequency of multiple blood meals (14.3% in our study), which could represent two independent transmission events, suggests that this phenomenon needs to be considered in models of malaria transmission. Additionally, the probability of infecting a human host during a single blood feeding, another parameter that determines vector species-specific contribution to transmission, has as upper limit the prevalence of sporozoites. In our setting, this prevalence varies in

different vector species, and this could be linked to variability in mosquito susceptibility to falciparum infection, as mentioned in the *Results* section; another explanation could be related to species-specific mosquito survival and age structure, as older mosquitoes are likely to have acquired infection in previous blood meals (see answer to previous comments).

As the species composition of local vector populations varies in the course of the rainy season (see *Results* section, and Dao et al. *Signatures of aestivation and migration in Sahelian malaria mosquito populations*. Nature 2014 for detailed discussion of vector species-specific population dynamics in another seasonal setting), these differences might impact infection propagation in the human population during the transition from dry to wet season and cause temporal variation in human force of infection during a single transmission season.

We have now modified this paragraph to mention mosquito age structure as one of the possible causes of variation in sporozoite prevalence, and to explicitly state these observations are relevant to understand local malaria epidemiology:

“Additionally, we observed that the most numerous species in our study area, A. coluzzii and A. gambiae s. s., differed in two key parameters that influence the transmission potential of mosquitoes: the likelihood of feeding on multiple individuals and the prevalence of sporozoites. There is evidence from membrane feeding experiments performed in Senegal (29) that A. gambiae s. s. mosquitoes might be more susceptible to malaria infection, although this association was not observed in another transmission study (30). Another possible explanation for the observed species-related difference in sporozoite prevalence could be variation in mosquito survival and consequently age structure. The higher frequency of falciparum infection in A. gambiae s. s. mosquitoes confirms, together with its relatively high abundance, the prime importance of this species for malaria transmission in the study setting. The contributions of the different Anopheles species to the mosquito infectious reservoir also depend on the number of potential parasite inoculations per mosquito-time: mosquitoes with multiple blood meals are more likely to infect more than one individual on a single night compared to mosquitoes with single-source blood meals, assuming sufficient quantities of sporozoites are inoculated during the probing of the

different hosts. The higher proportion of blood meals with multiple human DNA sources suggests that *A. coluzzii* mosquitoes also contribute significantly to the incidence of infection in humans. Importantly, as species composition of local vector populations varies in the course of the rainy season, these differences might impact the rate of infection propagation in human populations over the course of a single transmission season as relative abundances of the different vector species change.”

Reviewers’ comment

4. A map showing the location of houses and individuals sampled would be useful, including the distribution of those not sampled. Some description of the landscape and economy of the study area would be useful. This might explain why the sex ratio of the study population is so strongly skewed in favor of females. Were domestic animals present? If so, was screening done for non-human bloodmeals and what were the results?

Answer to Reviewers’ comment

The main economic activity in the health district of Saponé is subsistence farming and livestock keeping (Ouédraogo et al. *Malaria Morbidity in High and Seasonal Malaria Transmission Area of Burkina Faso*. PLOS One 2013). This is now mentioned in the *Methods* section:

“This study was conducted in Balonghin (health district of Saponé, Burkina Faso). The main economic activity in this area is subsistence farming and livestock keeping. Malaria transmission is seasonal, occurring between August and December following rainfall between June and September (39), and *falciparum* parasite prevalence is high, above 80% by 18S qPCR, during the transmission season (11).” (Lines 408 – 410)”

Regarding non-human blood meals, we observed that blood meals from only 8.2% (N=88) of tested mosquitoes did not amplify for human DNA. It is possible that some of these mosquitoes fed on domestic animals, however evidence from indoor collection studies demonstrates that *Anopheles gambiae* mosquitoes are highly anthropophilic (see **Figure 1** of a summary of different studies. Takken & Verhulst.

Host preferences of blood-feeding mosquitoes. Annu Rev Entomol. 2013). Importantly, since our main objective was to assess the distribution of mosquito bites in human populations, and since it is unlikely that non-human biting would divert bites from specific hosts, we do not expect this to bias our individual-level estimates. We have now mentioned the possibility of non-human biting in the *Discussion* section:

*“Of note, the age distribution of our study population (**Table 1**) might not reflect the true demography of the region. Whilst our results are in agreement with previous studies, we cannot exclude that a difference in age composition related to the fact that only houses with at least one child were included in the study may have influenced our heterogeneity estimates. Furthermore, we observed that blood meals from 8.2% of tested mosquitoes did not amplify for human DNA. Although it is possible that some of these mosquitoes fed on domestic animals, since it is unlikely that non-human biting would divert bites from specific hosts, we do not expect this to bias our individual-level estimates.” (Lines 320 – 324)*

Reviewers' comment

5. There seems to be an inconsistency between figure 2c and the sentence beginning on line 167 - "Within-household heterogeneity in mosquito exposure was observed: in 4/8, 14/15 and 7/7 houses with at least three study participants and five matched mosquitoes at the start, peak and end of the transmission season respectively, the most exposed individual was the source of more than 50% of mosquito blood meals (Figure 2c)." If figure 2c represents the 8 houses, then according to [reviewer WH's] inspection of the figure, the correct numbers should be 4/8 (we agree there), 3/8 (we are far off here - where does the denominator of 15 come from?), and 6/7 (though for the last house to be included in the numerator you'd need to specify "50% or more" in the interpretive sentence as in this house it seems that exactly 50% of bloodmeals came from a single individual).

Answer to Reviewers' comment

In **Figure 2c (now **2b**), only the 8 houses with at least 5 matched mosquitoes at the**

start of the transmission season were included; of these 8 houses, 6 also had 5 or more matched mosquitoes at the peak of the transmission season. The reason why we only present data for this subset of houses in this figure is because they had sufficient numbers of matched mosquitoes on which to base our calculations for at least two surveys. In the statement starting in line 167 (*Results* section), the survey-specific denominators comprise any houses with at least 5 matched mosquitoes in the corresponding survey. We have now modified this sentence to clarify this difference and also to mention that it corresponds to “50% or more”:

*“Within-household heterogeneity in mosquito exposure was observed (**Figure 2b**): considering data from houses with at least three study participants and five matched mosquitoes in single surveys, in 4/8, 14/15 and 7/7 households ~~with at least three study participants and five matched mosquitoes~~ at the start, peak and end of the transmission season respectively, the most exposed individual was the source of at least 50% of matched mosquito blood meals.”*